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REGULATION OF EXPRESSION OF THE Na^+/H^+ EXCHANGER (NHE1) GENE

by

JASON R. B. DYCK ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences (Pediatrics)

Department of
Medical Sciences
Edmonton, Alberta
Fall 1995

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JASON R. B. DYCK
Box 10, Site 7, R.R. 6
Edmonton, Alberta
T5B 4K3

Date: MAY 25, 1995

" I have little patience with scientists who take a board of wood, look for its thinnest part, and drill a great number of holes where the drilling is easy. "

Albert Einstein

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled REGULATION OF EXPRESSION OF THE Na^+/H^+ EXCHANGER (NHE1) GENE submitted by JASON R. B. DYCK in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences (Pediatrics).

Dr. Larry Fliegel (Supervisor)

R.C. Bleackley
Dr. Chris Bleackley

Bernard Lemire
Dr. Bernard Lemire

Peter Olley
Dr. Peter Olley

Dr. Luis Agellon

Dr. Bradford Berk

Date: MAY 25, 1995

DEDICATION

This thesis is dedicated to my family. To Mom, Dad, Sis, and Granny who supported me both financially and emotionally for far too long. To Doc and Bandit who took me away from it all for an hour every day, and to Michelle, who put her life on hold for longer than expected, always believed in me, and made it all worth while.

ABSTRACT

The aim of this work was to investigate the regulation of expression of the NHE1 isoform of the Na^+/H^+ exchanger. We examined the regulation of the level of exchanger message in the myocardium during ischemia and have studied the regulation of the NHE1 promoter during cellular differentiation and proliferation. In the myocardium, we have demonstrated that low-flow ischemia causes increased Na^+/H^+ exchanger message levels while perfusion at more reduced flow rates eliminated the increase. NHE1 message levels may act to compensate for ischemia induced acidosis whereas more severe ischemia may be detrimental to the myocardium and prevent the upregulation of the Na^+/H^+ exchanger. To examine the regulation of the promoter itself, we isolated and characterized the promoter/enhancer region of the mouse NHE1 gene. Deletion or mutation of an AP-2-like site contained within the promoter region resulted in a loss of most of the promoter activity indicating the importance of AP-2 in controlling NHE1 expression. AP-2 has been shown to increase during neuronal differentiation and since the Na^+/H^+ exchanger is believed to be important in cellular differentiation we examined regulation of the NHE1 expression during this event. We demonstrate a 10-fold AP-2 dependent increase in NHE1 transcription and an accompanying 3-fold increase in NHE1 activity in differentiating P19 cells. Both of these phenomena preceded major changes in cell morphology, suggesting that initiation of differentiation is linked to NHE1 gene expression. We have also examined factors important in regulation of expression of the NHE1 promoter in NIH 3T3 cells. Removal of serum from media reduced activity of the NHE1 promoter while three mitogenic agonists present in serum increased transcription. This suggested that the stimulation of growth may also stimulate the NHE1 gene. It was found that the activity of the NHE1 promoter was preferentially activated in the G2 phase of the cell cycle.

This indicated that NHE1 activation is consistent with a permissive role of the exchanger in cell growth and proliferation.

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LIST OF ABBREVIATIONS AND SYMBOLS

ATP	adenosine triphosphate
BCECF-AM	acetoxy methyl ester of 2'-7'-bis (2-carboxyethyl)- 5(6)-
bp	base pairs
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CaCl$_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
	carboxyfluorescein
CAT	chloramphenicol acetyl transferase
cDNA	complementary deoxyribonucleic acid
Cl$^-$/ HCO$_3^-$	chloride- bicarbonate
CO$_2$	carbon dioxide
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetate
EGTA	ethylene glycol bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
H$^+$	hydrogen ion
HCl	hydrochloric acid
HEPES	N-2-hydroxyethypiperazine-N'-2-ethanesulfonic acid
IgG	immunoglobulin G
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase
KCl	potasium chloride
kDa	kilodalton
MEM	minimal essential medium
MgCl$_2$	magnesium chloride

MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger ribonucleic acid
msec	millisecond
Na^+	sodium ion
$NaCl$	sodium chloride
NH_4Cl	ammonium chloride
NHE1	Na^+/H^+ exchanger - isoform #1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pHi	intracellular pH
PMSF	phenylmethylsulfonyl difluoride
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TE	Tris-EDTA
UV	ultraviolet
Vmax	maximal velocity
VSMC	vascular smooth muscle cells

DNA abbreviations

A	adenine
C	cytosine
G	guanine
T	thymine
U	uracil

CHAPTER ONE

Introduction

INTRODUCTION

Structure and Function of the Na^+/H^+ Exchanger:

In the majority of mammalian cells, cytoplasmic pH remains in a steady state due to the net effects of both acid producing and acid extruding processes. Most of the intracellular acid is produced as a result of metabolic by-products and the conversion of CO_2 to carbonic acid. The acid extruding processes involve Na^+ dependent and independent Cl^-/ HCO_3^- exchangers, an ATP-dependent H^+ pump and Na^+/H^+ exchange (1). How these regulatory mechanisms contribute to the maintenance of intracellular pH often varies between different cell types (2). Regardless of the different contributions, the cell is able to maintain pHi at approximately 7.2 with very narrow fluctuations. The Na^+/H^+ antiporter has been shown to be one of the most effective regulatory mechanisms in the cell which can maintain cytoplasmic pH against acid loads (3). This antiporter maintains intracellular pH and contributes to the survival of most, if not all, cells.

The Na^+/H^+ exchanger is a mammalian plasma membrane protein of approximately 110 kDa in size (4). It functions to remove intracellular H^+ ions in exchange for extracellular Na^+ ions with a 1:1 stoichiometry. The activity of the exchanger is driven by the transmembrane Na^+ gradient and requires no direct metabolic energy input. The exchanger was first identified in vesicles from rat kidney brush border membranes (5) and has since been shown to exist in almost every eukaryotic cell (6). This ubiquitous antiporter is controlled by a modifier site which can detect and respond to intracellular pH changes (7). Acidification increases the activity of the Na^+/H^+ antiporter and results in a rapid increase in pHi. Once pHi has been restored, the antiporter returns to its inactive resting level. Na^+/H^+ antiport occurs very rapidly and is an efficient method for maintaining optimal intracellular pH conditions.

The human NHE1 cDNA has been cloned and the protein has been predicted to contain 815 amino acids. The NHE1 isoform of the Na^+/H^+ exchanger family consists of two distinct domains. The first domain is the amphipathic NH_2 terminal domain which consists of 500 amino acids and contains putative N-linked glycosylation sites. The apparent molecular weight of this protein is larger than predicted from the amino acid sequence due to glycosylation (4). Site-directed mutagenesis has shown that NHE1 possesses both N- and O- linked glycosylation sites situated in the first extracellular region (8). Enzymatic deglycosylation, however, appears to have no effect on NHE1 function in the tissues studied thus far (9). The second domain is the c-terminal domain which lies in the cytoplasm of the cell. This region contains many phosphorylation sites and is involved in the regulation of the activity of the protein. Deletion of the cytoplasmic domain from NHE1 still allows for ion translocation, although the protein is no longer under normal regulation (10). Interestingly, the protein with the deleted c-terminus also shows certain characteristics which indicate that the modifier site is contained within this region. Finally, the functional unit required for Na^+/H^+ exchange has not yet been determined. It has been shown that NHE1 protein can exist as a dimer in the membrane (11) but ion translocation may depend on an oligomeric structure. Although much has been learned about NHE1, many unanswered questions still exist concerning this isoform of the NHE family.

Recently, several isoforms of the Na^+/H^+ exchanger have been identified in mammals, all of which originate from separate genes (4, 8). These isoforms are termed NHE1-4 and are believed to have 12 transmembrane domains and a large cytoplasmic tail consisting of up to several hundred amino acids. They have varying degrees of sequence identity from 40% in the transmembrane region to virtually no identity in the internal cytoplasmic region (12). The different isoforms must have evolved for specialized functions in various cell types. These isoforms possess different kinetic properties and are localized to specific tissues or specific regions of cells (13, 14). As mentioned previously only NHE1 has been shown to have a widespread distribution in almost every mammalian cell.

The ability to alter intracellular pH has implicated the Na^+/H^+ exchanger in mediating specific cellular responses. One example of this is cell proliferation. The most demonstrative events occur minutes after oocyte fertilization where there is a Na^+- dependent intracellular alkalinization (15). This occurs in concert with increases in DNA and protein synthesis, metabolic rates, and the initiation of cell division (16). It is hypothesized that the intracellular alkalinization regulates the subsequent biosynthetic events (16). Furthermore, serum and some of the mitogens it contains, can stimulate quiescent cells in culture to grow and divide (17). Serum-starved fibroblasts which have been subjected to alkalinization can induce mitosis (16). Also, Na^+/H^+ exchanger mutant fibroblasts do not alkalinize when exposed to serum mitogens and show reduced rates of DNA synthesis and cell growth in comparison to wild type fibroblasts (18). Thus it seems likely that the Na^+/H^+ exchanger plays an important role in cell growth and proliferation. The importance of the Na^+/H^+ exchanger in growth and proliferation is paramount when considering actively growing tumors. Tumor promoters such as phorbol esters have been shown to stimulate NHE1 (19). In addition, actively proliferating tumor cells are often more acidic than normal cells and inhibitors of the exchanger can abolish or reduce this proliferation (20).

Regulation of the Na^+/H^+ Exchanger Gene:

The regulation of the Na^+/H^+ exchanger (NHE1) gene has, until recently, received little attention from the research community. The human NHE1 cDNA has been cloned along with the cDNA from the rat and rabbit (21-24). The predicted amino acid sequences from these species display a strong sequence similarity to the human NHE1 cDNA (>90%; 22-24). The RNA blots from human, rat, and rabbit all indicate a message of approximately 5 kb in size (4, 21). The first studies which suggested regulation of the NHE1 gene involved the use of RNA blot analysis. The steady state levels of NHE1 mRNA was shown to increase under specific situations such as diabetes (25), acidosis (26-29), ischemia (30; Chapter 2, this document), and differentiation (31, 32). Since these changes in messenger RNA levels could not directly prove alterations in transcriptional activity, the promoter/enhancer region from the human and mouse gene have been cloned and sequenced (33, 34). This work has enabled a much more direct method to study the regulation of the promoter. Sequence analysis has provided information about putative cis-acting consensus regions which may be responsible for the regulation of the NHE1 promoter (33, 34). These consensus sites include regions which may be capable of binding some of the more well known transcription factors. These include C/EBP, AP-1 and AP-2 (33-35; Chapter 3, this document). Of these proximal regulatory elements, only AP-2 has been directly shown to interact with the NHE1 promoter and to be responsible for regulating the gene (35; Chapter 3, this document).

Until recently, the regulation of NHE1 activity has been thought to be governed by short-term changes in the activity of the transporter (4, 36). Now, however, there is strong evidence supporting long-term regulation at the level of the gene and during the pre-translational period. Most of this data has come from observing changes in the mRNA levels. These changes occur during a wide variety of cellular conditions such as acidosis (26-30; Chapter 2, this document), glucose stimulation (25), proliferation (37), and differentiation (31, 32). This work implies possible changes in transcriptional activity and suggests a strong rationale for the examination of the long-term regulation of NHE1.

One treatment which affects NHE1 expression is acidosis. Acidosis has long been known to increase NHE1 activity in specific cell types. To study how a decrease in intracellular pH affects mRNA levels in specific tissues, both animal models and tissue culture models have been used. Animals and cells were subjected to metabolic acidosis while specific renal cells were incubated with media of low external pH to cause intracellular acidosis. Krapf *et al.* (26) subjected rats to metabolic acidosis and measured mRNA levels in the proximal tubule (26). The proximal tubule was studied since it has been established that it is an important element in the adaptive response of the kidney to systemic acidosis (26). After 5 days of induction, the levels of NHE1 mRNA increased 1.8-fold during metabolic respiratory acidosis (26). Similarly, in LLC-PK$_1$ renal epithelial cells a 1.9-fold increase in NHE1 mRNA steady state levels were also reported after 48 hours of metabolic acidosis (38). Also, *in vitro* acid incubation of cultured rabbit renal tubule cells produced a 2 to 5-fold increase in NHE1 mRNA levels after a 24 hour incubation in acidic media. In NIH-3T3 fibroblasts, however, the identical treatment suppressed mRNA levels (27). The exact mechanism for these cellular differences is not known.

Both increased Vmax of Na^+/H^+ exchange and increased NHE1 message have been demonstrated in response to chronic metabolic acidosis in intact animals and renal cell lines (26, 27, 39-42). This effect is tissue specific in that it was shown in renal cortical tubule cells, in opossum kidney cells, and in LLC-PK_1 renal epithelial cells. The opposite, however, was seen in NIH 3T3 fibroblasts (27, 38). Whether this increase in mRNA is a result of increased transcription or decreased mRNA degradation is not known. It is possible that the gene or the message contains a cis-sequence which can bind a trans-element and increase transcription or decrease mRNA degradation (27). The presence or activation of this trans-element may be cell specific and therefore the acidic effect may only occur in certain cell types. Increased mRNA stability can account for some of the increased phosphoenolpyruvate carboxykinase mRNA seen in response to acidosis (43). Since alterations in mRNA levels due to an acidic environment has already been shown to occur in other proteins, this is the most likely scenario for the exchanger. It is not known, however, whether this occurs in other tissues as a response to chronic acidosis.

Rao *et al.* studied changes in mRNA levels in vascular smooth muscle cells (VSMC; 37). This study concentrated on the effects of vasoconstrictor and mitogen stimulation of NHE1 in vascular smooth muscle cells. Messenger RNA levels increased 25-fold in response to serum, platelet-derived growth factor and fibroblast growth factor. When VSMC were treated with a hypertrophic agonist, however, no increase in exchanger mRNA was reported

(37). The responsiveness of NHE1 to hyperplastic agonists indicates that mRNA levels are regulated in a growth dependent manner (37). Since the proliferation of the VSMC seems to induce increases in NHE1 mRNA levels, it is possible that the gene is also involved in the long-term response. Further experiments are needed, however, before transcriptional activity can be implicated in this regulation.

Another study involving VSMC investigated the effects of glucose on NHE1 mRNA levels. Glucose was chosen since diabetes mellitus is characterized by the development of hyperglycemia (25). Experiments done *in vivo* concentrated on the effects of elevated extracellular glucose concentrations on the levels of NHE1 mRNA. When VSMC were exposed to high concentrations of glucose (20 mM) for 24 hours, the result was a 3-fold increase in NHE1 steady state mRNA levels. Also, glucose dependent increases in NHE1 activity could be inhibited by Actinomycin D suggesting elevated mRNA levels were due to increases in gene transcription (25). These results suggest that abnormal glucose levels may lead to vascular injury due to the result of increased NHE1 promoter activity. The exact mechanism of how elevated glucose levels affects the NHE1 promoter has not been investigated.

The Na^+/H^+ exchanger is involved in cell proliferation and differentiation (31). During cell proliferation the exchanger is responsible for an elevation of intracellular pH. In some cell types this pH change has been shown to play an important permissive role in growth (36). Cell differentiation, however, results in more drastic alterations in growth patterns and involves the activation of many key genes. How the Na^+/H^+ exchanger gene is regulated and involved during this process remains unknown. Rao *et al.* (31) examined the regulation of Na^+/H^+ exchanger expression and its role in retinoic acid induced differentiation of HL 60 cells. Immediately prior to differentiation into granulocyte-like cells, the activity of the Na^+/H^+ exchanger increases and remains elevated well into differentiation (31, 44). There is an 18-fold increase in NHE1 mRNA levels as well as a 7-fold increase in protein levels (31). Transcriptional activity of differentiating HL 60 cells using nuclear run-on assays was also examined (31). After 72 hours of retinoic acid treatment there was a 8.3-fold increase in NHE1 promoter activity (31). This study provided, for the first time, direct evidence to support the hypothesis that the NHE1 gene could be upregulated during specific cellular events.

The recent cloning of the human NHE1 gene (29) has provided more insight into the possible regulatory elements responsible for controlling NHE1 transcription. Lying just upstream of the human NHE1 TATA box are a number of putative cis-elements which may be involved in regulating the NHE1 promoter. These sites include four GC boxes, two CAAT boxes, five CACCC boxes, three AP-1 sites, and a cyclic AMP response element (33). Sequential deletions of the promoter region were performed from a CAT reporter plasmid and the changes in CAT activity analyzed (34). The results indicate that regions exist which positively regulate the human NHE1 gene and that various tissues contain different NHE1 regulatory elements (34). Furthermore, a region of DNA which binds an as yet unidentified trans-acting protein is essential for NHE1 transcription in hepatic VSM tissue (34). Also, AP-1 has been shown to increase during acid incubation of mouse proximal tubule cells which can bind to the NHE1 AP-1 site and possibly regulate transcription (29). The search for the transcription factors involved in the regulation of the NHE1 gene has just started and will undoubtedly reveal many more elements capable of controlling the NHE1 gene.

During the regulation of a gene, it is usually safe to assume that some form of control is exerted through trans-acting factors binding to the promoter/enhancer region of the gene. Also, there must be some physiological event which induces or regulates these trans-factors thereby stimulating transcription from the NHE1 gene. The mouse NHE1 gene has been reported to be controlled by the transcription factor AP-2 (35; Chapter 3, this document). Also, retinoic acid induced differentiation has been shown to increase transcription of the NHE1 gene in HL 60 cells (31). In addition, it has been demonstrated that retinoic acid induced differentiation of the embryonal carcinoma cell line, P19, results in increased levels of AP-2 protein and mRNA (45). From this information we propose that a series of events occurs during differentiation of P19 cells which results in increased NHE1 promoter activity. The retinoic acid treatment of P19 cells initiates differentiation and increases AP-2 levels (45). Elevated AP-2 levels may then be able to induce transcription from the NHE1 gene. If our hypothesis is correct, it is possible that there may be a direct link between the NHE1 and cellular differentiation.

Since the cloning of the human NHE1 gene, many questions have remained unanswered in regards to how the promoter is regulated. Much of the recent work on the NHE1 gene regulation has involved the measurement of steady state mRNA levels during specific cellular events. These studies, however, tell us little about how the gene itself is regulated. Many studies have shown the involvement of NHE1 in cell growth and proliferation (36, 37, 46, 47). The effects of mitogenic stimulation have been well documented at the protein level (37, 46, 48, 49), however, the involvement of the gene itself has not been investigated. It is our hypothesis that the gene many be regulated during mitogenic stimulation. Also, we propose that this regulation is due to secondary effects brought on by re-introduction of the cell into the cell cycle. It is possible that different phases of the cell cycle require various levels of NHE1 protein, and these levels may be, in part, regulated by the gene. Since a dividing cell seems to require alterations in intracellular pH, it is our belief that this can occur through changes in gene expression. An actively dividing cell may require increased NHE1 activity and part of this increase may come via increases in promoter activity resulting in increased protein levels.

The Na^+/H^+ Exchanger in the Myocardium:

In the myocardium the Na^+/H^+ exchanger is activated over a lower pH range than some cell types (50, 51). This suggests that the exchanger is more likely to be involved in pH regulation during an acute acid challenge. Some basic characterization studies have shown that the Na^+/H^+ exchanger exists on the heart sarcolemma (52-54) and has qualities which vary in similarity to the exchanger in other tissues (54-56). In the myocardium, the regulation of intracellular pH is important for two major reasons. Firstly, intracellular acidosis is important during the contractile failure associated with ischemia (57, 58). With a fall in intracellular pH, contractility can decline greatly and significantly affect heart function. The second reason why the regulation of internal pH is of special importance is that the Na^+/H^+ exchanger is believed to be important in modulating the cardiac response to reperfusion (12, 59-61). During ischemia and reperfusion of the myocardium, cell damage, necrosis, and arrhythmias can occur. Much of the damage of these episodes is associated with reperfusion and not with the ischemic phase (62, 63). The mechanism by which this occurs may involve the Na^+/H^+ exchanger (12, 59-61). Ischemia results in increased intracellular protons. These protons accumulate inside the myocytes until the reperfusion phase when the Na^+/H^+ exchanger becomes active and removes them. This results in a rapid increase in intracellular Na^+. The increase in intracellular Na^+ activates the Na^+-Ca^{2+} exchanger which causes increased intracellular Ca^{2+} and subsequent damage to the myocardium. This series of events is sometimes referred to as the 'pH paradox'.

Evidence suggests that the isoform present in the myocardium is the NHE1 type. NHE1 cDNA clones have been isolated from the myocardium of a variety of mammalian species (24, 64, 65). They show no differences in comparison to other NHE1 clones from other tissues. The presence of the 5 kb NHE1 isoform in mRNA from intact hearts has also been shown in a number of other studies by RNA blot analysis (24, 65-67). The mammalian myocardium consists predominantly of myocytes, however, a significant amount of other cell types exist. Primary cultures of isolated myocytes were examined for the presence of NHE1 in a population of myocytes free of other cell types. Both RNA blot analysis and reverse transcriptase polymerase chain reaction demonstrated the presence of the NHE1 isoform (64). These results clearly show that NHE1 is expressed in cardiac myocytes. Regulation of the NHE1 message in the myocardium is only recently being studied. When rabbit hearts are subjected to relatively short periods of ischemia there is a small increase in NHE1 mRNA levels (5 kb) and a larger increase in a related isoform (3.8 kb) of the message (67). The identity of the 3.8 kb isoform and its relationship to Na^+/H^+ exchange is unknown. Our hypothesis is that the level of myocardial Na^+/H^+ exchanger mRNA is increased in response to ischemia and the resulting acidic environment. If there is an adaptive mechanism in the myocardium in response to ischemia, it could play a very important physiological role in protecting the heart from excess acid. Greater Na^+/H^+ exchange in response to ischemia or angina, however, could be detrimental during reperfusion of the myocardium. Future studies may investigate this important paradox.

Summary and Rationale:

Our initial experiments will concentrate on the myocardium during ischemia. Since other studies have already shown increases in NHE1 mRNA levels in response to acid (26, 27, 39-42), we will attempt to determine if the acidic environment produced during ischemia could produce similar effects in the myocardium. It is possible that a pH sensitive regulatory mechanism is able to either increase transcription or increase the stability of the NHE1 mRNA. We will also try to determine whether the severity of ischemia alters the levels of NHE1 mRNA. Ours studies on the steady state levels of NHE1 mRNA may eventually lead to the discovery of a novel ischemia induced transcription regulatory mechanism. Because of this, we have set out to gain a better understanding of the regulation of the NHE1 gene. Since the key regulatory elements involved in gene regulation may lie upstream of the transcriptional start site, the NHE1 promoter/enhancer region will be characterized. Direct study of the regulation of the NHE1 gene requires understanding of the various factors involved in the activation of the gene. We will examine regions of the mouse NHE1 gene which may be involved in the binding of specific transcription factors. These experiments will provide significant information regarding the regulation of the NHE1 gene. Furthermore, these studies may allow for the identification of specific cellular events which require increased NHE1 transcription. It has already been shown that in some cell types dramatic increases in NHE1 mRNA levels accompany cell differentiation (31, 32). We will examine a specific model of cell differentiation and determine how the NHE1 gene is regulated during this event. If the induction of the NHE1 gene can be linked to differentiation, it is also possible that a similar mechanism is involved in cellular proliferation. As discussed earlier, cellular proliferation may be responsible for altering the activity of the NHE1 gene. Mitogenic stimulation of some cell types has been shown to result in increases in NHE1 levels. Since a dividing cell seems to require alterations in intracellular pH, it is our belief that this can occur through changes in gene expression which are at least partially mediated through increased transcription. We will attempt to identify how the Na^+/H^+ exchanger gene is involved in this process.

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CHAPTER TWO

Induction of Expression of the Na+/H+ Exchanger in the Rat Myocardium

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Jason R. B. Dyck, Thane G. Maddaford, Grant N. Pierce and Larry Fliegel.

INTRODUCTION

The regulation of internal myocardial pH is of special importance to the functioning heart. Typically resting intracellular pH is near 7.2 but drops dramatically during ischemia causing depression of myocardial contractility. The negative inotropic effect of acidosis has been demonstrated in several cardiac preparations ranging from cardiac muscle fibers (1) to isolated perfused heart (2) and the rabbit heart *in vivo* (3). The lowered pHi depresses contractility by acting on a number of steps of excitation-contraction coupling (reviewed in 4).

The Na+/H+ exchanger is responsible for regulating myocardial intracellular pH with at least two other transporters: a Cl^-/HCO_3^- exchanger and a Na^+/HCO_3^- cotransporter (5, 6). The different transporters have specialized roles with relatively little overlap. The Cl^-/HCO_3^- exchanger can be activated by a more alkaline intracellular pHi (7, 8), and may serve to acidify the cell when needed. The Na+/H+ exchanger, which is activated by acidic pH (9), helps the cell to recover from a strong acid challenge. The Na^+/HCO_3^- cotransporter also exports acid equivalents by importing bicarbonate together with Na+, and aids the Na+/H+ antiporter during recovery from acidosis. Its contribution relative to Na+/H+ exchange varies from 20-40% depending on the cardiac preparation (6, 7). Because of the steep activation curve of Na+/H+ exchange with lowered intracellular pH (9), the Na+/H+ exchanger likely plays a more important role in recovery from acute acid load such as that induced by ischemia. Only a small acidic shift (by 0.1 to 0.2 pH units) in the pH dependence curve of Na+/H+ exchange is required to raise the activity of the Na+/H+ exchanger. This causes cytoplasmic alkalinization (9, 10). In addition, exposure to alpha-adrenergic agonists causes activation of the exchanger suggesting the exchanger and intracellular pH regulation may be important in alpha-adrenoreceptor induced positive inotropy (10, 11).

The Na^+/H^+ exchanger is also involved with damage associated with ischemia and reperfusion of the myocardium. Hearts rendered ischemic for long periods of time do not completely recover their contractile activity upon reperfusion. Damage associated with these episodes often occurs with reperfusion of the myocardium and not with the initial ischemic period (12, 13). During ischemia, acid accumulates in the cell. Intracellular Na^+ also increases dramatically during the ischemic period (14, 15). However, the low intracellular pH only stimulates Na^+/H^+ exchange a limited amount since a low extracellular pH also occurs and reduces exchanger activity. Once reperfusion is started, extracellular pH soon returns to normal and removes inhibition of the Na^+/H^+ exchanger. The further rise in intracellular Na^+ induced by the antiporter is believed to activate Ca^{2+} influx via the Na^+/Ca^{2+} exchanger. This results in Ca^{2+}-overload and cell death or arrhythmias (16-21). Several studies also showed that intracellular Na^+ levels increase significantly during both ischemia and reperfusion and that inhibiting the antiporter with amiloride and related analogs can reduce Na^+ accumulation (19-21). The inhibition of the Na^+/H^+ exchanger prevents reperfusion-induced increases in Na^+ and Ca^{2+} concentrations, which reduces reperfusion-induced injury (17, 19-21).

There appears to be a physiologically adaptive mechanism by which some mammalian cell types are able to upregulate this acid-removing transporter in response to chronic acid load. Chronic metabolic acidosis, induced by addition of NH_4Cl to drinking water, increased the Vmax of

Na+/H+ exchange in several types of renal membrane vesicle preparations (22, 23). Others have also shown similar effects on the renal cortex and LLC-PK_1 renal epithelial cells. Chronic metabolic acidosis induced by NH_4Cl feeding or treatment of cells in culture with low pH media, results in increased NHE1 mRNA levels (24-26). The same type of treatment decreases the mRNA abundance of the Na+/H+ exchanger in fibroblasts, demonstrating the tissue specificity of the effect (24).

We have earlier demonstrated that the amiloride sensitive Na+/H+ antiporter is present in the rabbit myocardium (27, 28). There is a 5.0 kb message corresponding to the NHE1 message and a related 3.8 kb message that hybridized under low stringency. When rabbit hearts are subjected to relatively short periods of ischemia there was an increase in the 3.8 kb message (28). The 3.8 kb message was likely the product of another gene and its exact identity is unknown. In the present study, we examine the regulation of the cardiac Na+/H+ exchanger NHE1 isoform mRNA in response to long periods of ischemia in the isolated perfused rat heart. In addition we test the effect of chronic acidosis on the activity of the Na+/H+ exchanger in isolated myocytes. The results suggest that increased expression of the 5.0 kb NHE1 message does occur in the isolated perfused heart in response to 3 hour periods of ischemia. Increased activity of the exchanger can also be induced by external acidosis in primary cultures of isolated myocytes. The results suggest that the Na+/H+ exchanger could increase in activity in the human myocardium in response to ischemia. This could have important clinical consequences.

MATERIALS AND METHODS

Heart Perfusions

Male Sprague Dawley rats weighing 350-400g were obtained from the Animal Care Facilities, University of Manitoba, Winnipeg. Animals were sacrificed by decapitation and their hearts rapidly excised and perfused in a non circulated, retrograde fashion according to the Langendorff method (29). Hearts were constantly perfused with the aid of a peristaltic pump at a rate of 10 ml/min. The perfusate was composed of (in mM): NaCl, 140; KCl, 6; $MgCl_2$, 1; $CaCl_2$, 1; dextrose, 10; HEPES, 6 (pH 7.4, 37°C and was bubbled constantly with 100% 0_2). Myocardial contractile force was measured by attaching the apex of the heart to a force transducer which was connected to a Linearecorder Mark VII WR3101 polygraph recorder (Graphtec, Irvine, CA). The heart was paced at a rate of 200 beats/min at 200% of threshold with 9 msec stimulation duration. All hearts were allowed to stabilize their contractile activity for at least 15 minutes before initiating the ischemic challenge. To render the heart ischemic, the pump flow rate was reduced to 3, 1 or 0 ml/minutes. During ischemia, a Plexiglas cover was placed over the perfusion chamber and the temperature and humidity maintained, as described (19, 20). The ischemic duration was 3 hours. Reperfusion was initiated by increasing the flow rate to 10 ml/minutes. Reperfusion was for 5 min. Hearts were immediately taken off the perfusion apparatus, trimmed quickly of visible aortic tissue, blotted and frozen rapidly in liquid N_2. Hearts were weighed quickly while frozen, then stored at -80°C until ready for use.

RNA isolation and analysis

Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim and Bethesda Research Laboratories. The plasmids pTZ18R and 19R were from Pharmacia LKB Biotechnology Inc. [^{32}P]ATP was obtained from New England Nuclear. Immobilon-N filters were from Millipore. All chemicals were of the highest grade available.

Poly (A$^+$) RNA was prepared from isolated perfused rat hearts using a modified procedure similar to that described earlier (27, 28). For each RNA blot RNA was isolated from different hearts, and for each point of a Northern RNA was isolated from tissue of one heart perfused as described. Ten ug of Poly (A$^+$) RNA was applied to each lane of the RNA blots. The RNA blot was probed with [^{32}P] labeled random primed cDNA (BRL). The fragment used for labeling was nucleotides 1-688 of the rabbit cardiac cDNA clone (27) corresponding to the coding region of this clone. To confirm that the RNA samples were of the same quantity and quality, all RNA blots were stripped and reprobed in control experiments using a cDNA fragment containing rat glyceraldehyde 3-phosphate dehydrogenase. Exposure times were 16 hours for Northerns probed with Na^+/H^+ exchanger probe and 4 hours when Northerns were reprobed with rat glyceraldehyde 3-phosphate dehydrogenase. Hybridization and washes were as described earlier (27, 28), with blots being routinely washed with 1 x SSC at 58°C (medium stringency). Scanning densitometry was with a Joyce Loebl Chromoscan 3. The ratio of Na^+/H^+ antiporter message to glyceraldehyde 3-phosphate dehydrogenase message was used for comparisons between control and ischemic groups. Statistical significance was determined with a Mann-Whitney U-test. RNA blots were the mean of 4 experiments, all other experiments were the means of at least 5 experiments.

Primary cultures of isolated myocytes and internal pH measurement

Primary cardiocyte cultures were prepared from neonatal rats essentially as we have described earlier (30). Briefly, hearts were removed from 6 day old rats under aseptic conditions and ventricles minced to small size. The tissue was digested with a series of treatments with 0.1% collagenase (Worthington, Biochemical Corp.). To selectively remove nonmyocardial cells a differential attachment procedure was used. Dissociated cells were incubated in Corning T-75 culture flasks at 37 °C in a humidified atmosphere (5% CO_2, 95% air) for a 20 minute period. During this time nonmyocardiocytes (fibroblasts, endothelial cells, and smooth muscle cells) attach and the majority of the myocytes remain in suspension. Subsequently myocardiocytes were remove and plated at 1 X 10^5 cells/cm^2. They were maintained in medium containing DMEM/F12 supplemented with 10% fetal bovine sera and containing, 10 μg/ml transferrin, 10 μg/ml insulin, 10 ng/ml selenium, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mg/ml BSA, 5 μg/ml linoleic acid, 3 mM pyruvic acid, 0.1 mM MEM-nonessential amino acids, 10% MEM vitamins and 30 mM HEPES pH 7.4. In some experiments myocytes were maintained in the same medium at pH 6.9 for 18 hours before intracellular pH measurement. Examination of neonatal myocytes kept in culture for up to 2 weeks by light microscopy or immunostaining revealed cultures essentially free of nonmyocardial cells. The myocardiocytes contained myocyte like morphology and characteristic cross-striations. Actin immunocytochemistry showed the characteristic myocyte like structure (not shown). For measurement of intracellular pH myocytes were grown on coverslips. The acetoxy methyl ester of 2'-7'-bis (2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) was used to measure internal pH. Coverslips were placed in a modified cuvette with a coverslip holder and a stirring bar. BCECF-AM (2 μg/ml) was incubated with cells for 5 minutes in a solution containing 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.5 mM Glucose, 10 mM HEPES, pH 7.4. Fluorescence measurements were made on a Shimadzu RF-5000 spectrofluorophotometer. Intracellular pH was measured using the dual excitation single emission ratio technique. Excitation wavelengths were at 490 and 440 nM with emission at 545 nM. This allows measurement of intracellular pH that is independent of cell concentration and loading (31). A calibration curve for intracellular dye was generated by the nigericin method (31). BCECF-loaded cells were placed into high KCl containing buffer with 10 μM nigericin. This clamps the H^+ gradient and sets internal pH equal to external pH. To measure initial rates of proton extrusion after an acid challenge the NH_4Cl prepulse method was used. Cells were incubated with buffer containing 15 mM NH_4Cl for 5 min. They were washed 3 times in Na^+ free buffer that contained 135 mM N-methyl-D-glucamine instead of NaCl. A solution containing 3 mM NaCl (and N-methyl-D-glucamine) was then added and the initial rate of rise of internal pH was measured over the first 40 seconds.

The pH_i decrease by the removal of NH_4^+ (ΔpH_i) was used to calculate the cell buffer capacity (ß), using the formula:

$$ß= \frac{[NH_4^+]_i}{\Delta pH_i},$$

where $[NH_4^+]_i$ was calculated from the pH just prior to NH_3/NH_4^+ removal, the extracellular $[NH_4^+]_i$, and the extracellular pH.

$$\Delta pH_i = \text{final} - \text{initial}$$

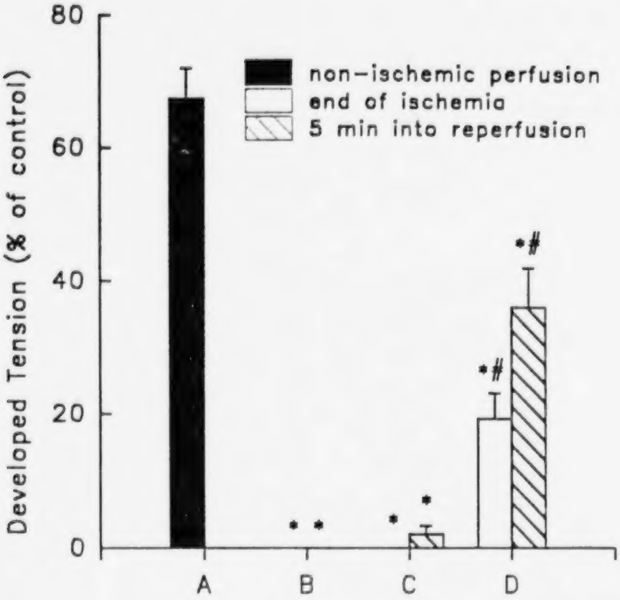
$$[NH_4^+]_i = \frac{NH_4Cl \bullet 10^{(pKa-pHi)}}{(1 + 10^{(9.21-pHo)})}, \quad pka=9.21$$

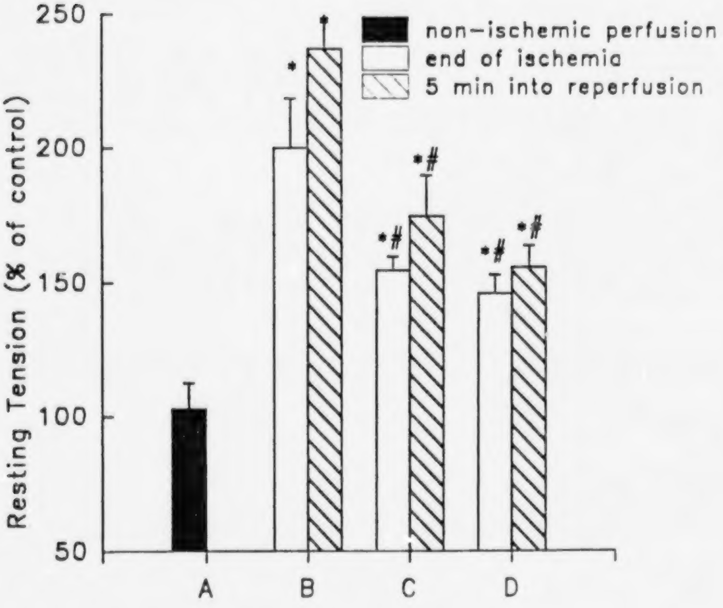
RESULTS

Hearts were perfused continuously at 10 ml/minutes for 3 hours or subjected to ischemic challenge for the same duration. The effects of this protocol on active developed tension and resting tension in these hearts are shown in Figure 2-1. Over the 3 hours of perfusion, active tension dropped about 28% from the start of the experiment. The active tension was not optimal because of a concomitant drop in resting tension (~15% from the start of the experiment). Resting tension could have been adjusted upwards in the control hearts to produce a more accurate reflection of optimal developed tension. However, it was decided that these values would not be manipulated to ensure a close and accurate comparison with the ischemic challenged hearts which were not adjusted throughout the experimental protocol. As shown in Figure 2-1, global, no-flow ischemia for 3 hours resulted in no active developed tension at the end of ischemia and no recovery of active developed tension during reperfusion. Low-flow ischemia (1 ml/minute) for 3 hours also produced no active tension at the end of ischemia but did allow for a slight recovery in tension generation during reflow (2% of the pre-ischemic value). A higher rate of low-flow ischemia (3 ml/minute) allowed for active developed tension to be maintained at ~20% of the pre-ischemic levels during the ischemic insult and further recovery to ~40% during the reperfusion phase.

Resting tension is often used as an indicator of contracture formation in the myocardium after ischemic challenge (16, 19, 20). Global, no-flow ischemia for 3 hours resulted in large rises in resting tension during both the ischemic and reperfusion phases (Figure 2-1). Low flow ischemia of either 1 or 3 ml/minute produced smaller increases in resting tension during both perfusion phases in comparison to the total, no-flow ischemic insult.

Figure 2-1. **Developed tension and resting tension in perfused rat hearts.** Developed tension (upper graph) and resting tension (lower graph) in hearts perfused for 3 hours at 10 ml/minute (A; non-ischemic), 0 ml/minute (B), 1 ml/minute (C) or 3 ml/minute (D). For the latter three groups, tension measurements are presented separately for the end of the 3 hours of ischemia and at the end of the first 5 minutes of reperfusion. Values represent the mean (± SE) for at least five separate samples. * denotes significant difference from respective non-ischemic value ($P<0.05$). # indicates significant difference from respective value in B group. Absolute values for active developed tension and resting tension were 11.1 g/g wet weight and 5.4 g/g wet weight, respectively. These values are similar to those reported earlier (39). Data obtained from the laboratory of Dr. G. N. Pierce.


80
60
40
20
0
Developed Tension (% of control)
non-ischemic perfusion
end of ischemia
5 min into reperfusion
A
B
C
D
* *
*
*
*#
*#


250
200
150
100
50
Resting Tension (% of control)
non-ischemic perfusion
end of ischemia
5 min into reperfusion
A
B
C
D
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Poly(A)+ RNA was isolated from the heart perfused under the condition described above. There were no significant differences in the yield of either total or poly(A)+ RNA from any of the groups of hearts. Figure 2-2a shows typical results of RNA blot analysis of poly(A)+ RNA from isolated perfused rat hearts probed with a fragment from the coding region of the NHE1 isoform of the rabbit Na+/H+ exchanger. Ten µg of poly(A)+ RNA was added to each lane and the RNA blot was washed with 1 x SSC at 58°C. Lane 1 is mRNA from hearts subjected to control aerobic perfusion of 10 ml/minute while lanes 2-4 are mRNA from hearts subjected to 3, 1 or 0 ml/minute of ischemia as described in the materials and methods. The blot was reprobed with cDNA encoding for glyceraldehyde 3-phosphate dehydrogenase (Figure 2-2b). The results showed that the NHE1 probe hybridized with a 5 kb message coding for NHE1 similar to results described earlier. When comparing the amounts of NHE1, lane 1 the control, contains an intermediate amount of message hybridizing to the NHE1 probe. Lane 2 (3 ml/minute) shows an increased amount of NHE1 message relative to lane 1. This increase was consistent in 4 separate independent experiments. In contrast the amount of exchanger message in lanes 3 and 4 often appeared to decrease. To more accurately quantify the changes that were occurring we examined 4 sets of independent experiments for each group. Autoradiograms of RNA blots were analyzed using scanning densitometry and the ratio of Na+/H+ exchanger to glyceraldehyde 3-phosphate dehydrogenase was measured. The results are shown in Figure 2-3. The mean change in the Na+/H+ exchanger/glyceraldehyde 3-phosphate dehydrogenase ratio in comparison to the control was +59, -18, and -24 for the groups of ischemia treated hearts of 3, 1 and 0 ml/minute respectively. The difference was significantly different from the control value for the 3 ml/minute group only.

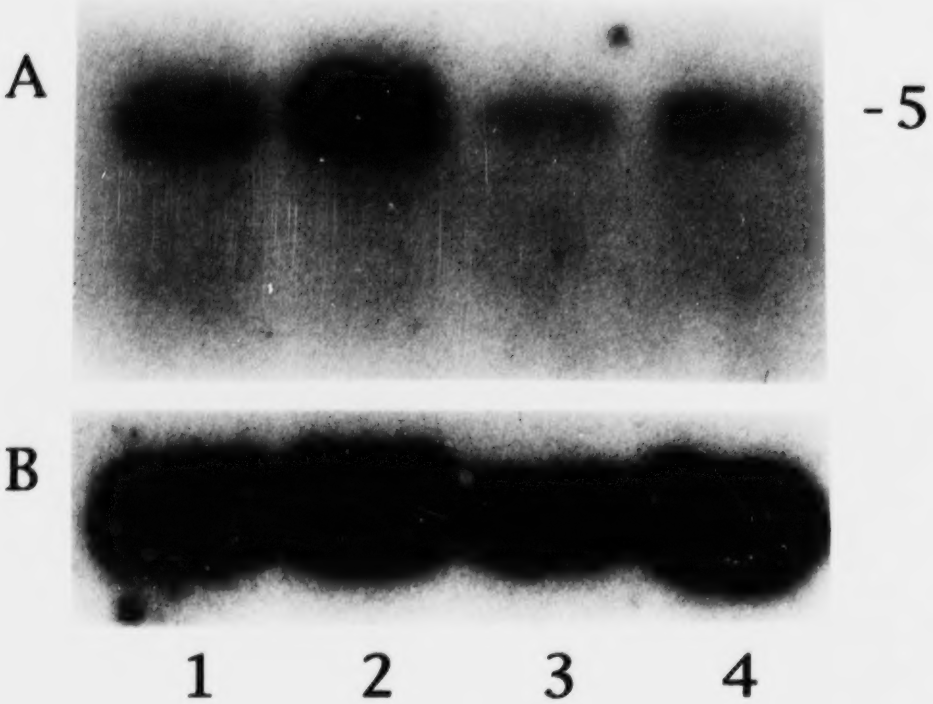

Figure 2-2. **RNA blot analysis of poly(A)$^+$ RNA from isolated perfused rat hearts.** Hearts were perfused as described in the "Materials and Methods" and subjected to either control aerobic perfusion (10 ml/min.) (Lane 1) or 3 hours of varying degrees of ischemia (Lane 2, 3 ml/min., lane 3, 1 ml/min. lane 4, 0 ml/min. Poly(A)$^+$ RNA was isolated and separated by size on formaldehyde agarose gels. After transfer to Immobilon-N, mRNA was probed with residues 1-688 of the rabbit cardiac Na$^+$/H$^+$ exchanger (27) and subsequently with a rat glyceraldehyde 3-phosphate dehydrogenase probe. Sizes were estimated based on the positions of the ribosomal subunits run simultaneously on the same gel. The relative positions of the 5.0 kb, 28S RNA subunit is noted. (A) Exposure of Northern after hybridization with NHE1 probe as described in the "Materials and Methods". (B) Exposure of the same Northern after being stripped and reprobed with a fragment of rat glyceraldehyde 3-phosphate dehydrogenase as described in "Materials and Methods".

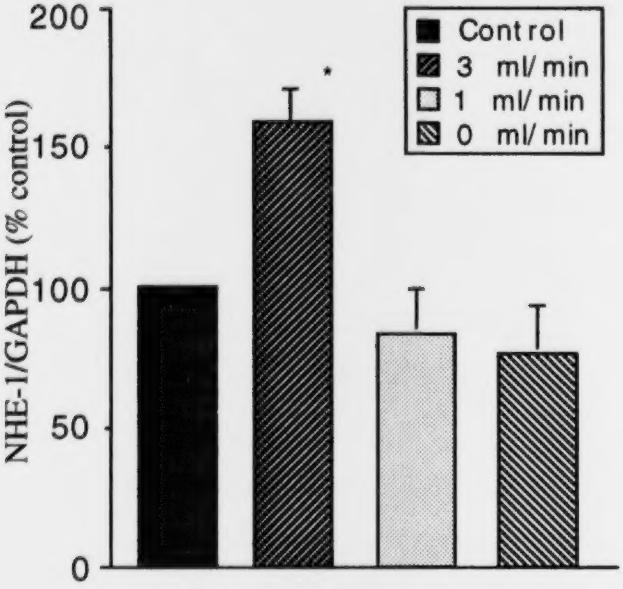

Figure 2-3. **Analysis of NHE1/glyceraldehyde 3-phosphate dehydrogenase ratios of RNA blots.** Blots were analyzed by scanning densitometry as described in the "Materials and Methods." RNA blots were probed with NHE1 probes and stripped and reprobed with glyceraldehyde 3-phosphate dehydrogenase. Autoradiograms were scanned and the ratio of the exposures were used to compare the experimental levels of the NHE1 to the control perfusions. All data represent the means (± SE) of four series of independent experiments. * Designates significantly different from control values at $P< 0.01$.

To examine the possible effects of acidosis on the activity of the Na+/H+ exchanger we used primary cultures of isolated myocytes prepared as described earlier (30). The rate of recovery from an acute acid load was measured in two groups. One group was treated with chronic low external pH in medium of pH = 6.9 for 18 hours, while the control group had medium of pH 7.4. Intracellular pH was measured using BCECF and an acute acid load was induced with ammonium chloride. Figure 2-4 shows the tracing of a typical experiment in the control group. The rate of recovery from ammonium chloride induced acid load of an experimental group (E) is superimposed. It is apparent that the experimental group recovered at a much greater rate than the control (C) group. There was no difference in the buffering power of the two groups (control= 29.16 ± 2.59 mM/pH unit; experimental= 30.08 ± 2.17 mM/pH unit) of cells and no difference in the amount of initial alkalinization and subsequent acidification induced by ammonium chloride in the two groups. The recovery from acid load was inhibited by the amiloride analogue hexamethylamiloride (not shown). The summary of the differences in the initial rate of recovery from an acid load is show in Figure 2-5. The acid treated cells recovered at a rate over 2-fold greater than control cells.

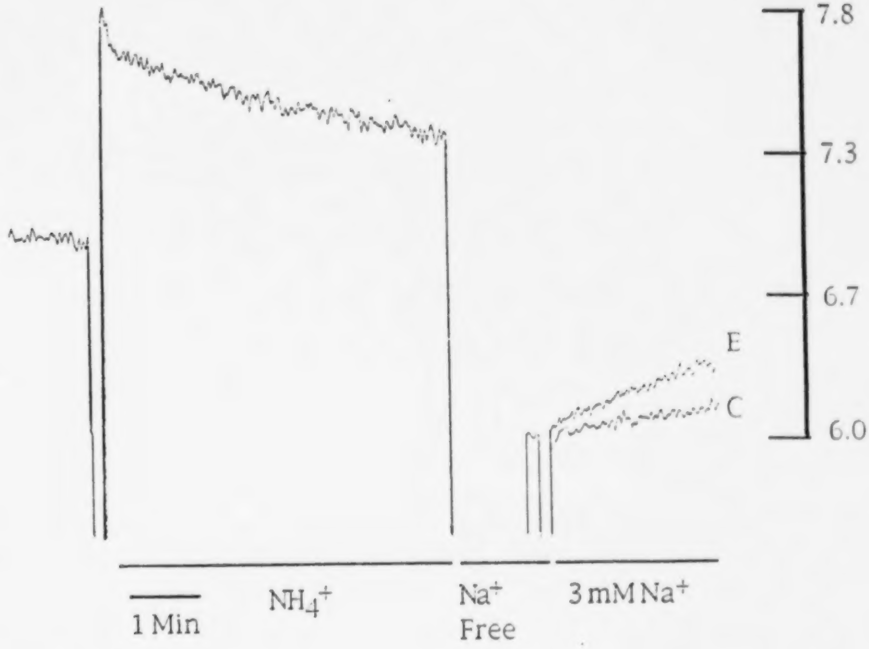

Figure 2-4. **Recovery from acute acid loads of primary cultures of isolated myocytes.** Cells were prepared and intracellular pH was measured as described in "Materials and Methods." Ammonium chloride prepulse was used to induce acute acidosis. The intracellular pH of cells grown in normal media is shown (C) and the recovery is in 3 mM NaCl. The recovery from an acute acid load is also shown, from an experimental group (E) treated with chronic low external pH (= 6.9) for 18 hours.

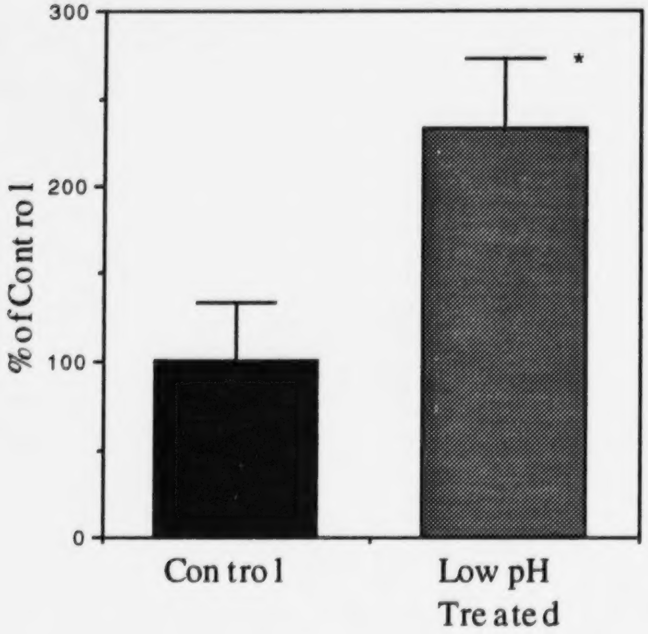

Figure 2-5. **Initial rates of recovery from an acute acid load by primary cultures of isolated myocytes.** Control cells and cells treated with low pH were examined as described for Figure 2-4. The initial rate of recovery is in the presence of 3 mM NaCl. Each group represents the means (± SE) of at least 6 independent experiments. * Designates significantly different from control values at P< 0.01.

DISCUSSION

We have examined the Na+/H+ exchanger message in intact isolated perfused rat hearts. A number of studies have suggested that the Na+/H+ exchanger gene can be activated by environmental factors that reduce intracellular pH (23-26). The Vmax of Na+/H+ exchange activity and the level of the NHE1 isoform of the Na+/H+ exchanger message is increased by chronic external acid load (23-26). This effect on Na+/H+ exchanger expression has been noted in the kidneys of treated animal models and in a number or renal cell lines. The increase in message is tissue specific in that fibroblasts are not affected by the same types of treatments (22). The present study examined the Na+/H+ exchanger message in the intact myocardium that was perfused for 3 hours. Though the times involved are relatively short, recent studies on isolated myocytes have shown that a number of different messages can be induced by various stimuli within much shorter time periods (32).

In our earlier study (28) we noted the presence of a smaller 3.8 kb message in rabbit hearts that hybridized to the rabbit NHE1 probe. We showed that the 3.8 kb message related to NHE1 can be increased in amount by perfusion for even shorter time periods than in the present study. In this series of experiments we did not observe the presence of this isoform. Hybridization at low stringency was required to observe the presence of this message in the rabbit myocardium (28). Since our probe was from the rabbit NHE1 (27) and we were examining rat message, it is likely that the probe does not cross hybridize to this other related message. Alternatively, the 3.8 kb message may not be present in this species. We also noted a small increase in the level of the 5 kb message with ischemia followed by reperfusion. This minor increase suggested that the 5 kb message could be altered in the myocardium and led to the present series of experiments.

The results of the present study show that low-flow ischemia can cause an elevation of the 5 kb message that codes for the Na+/H+ exchanger (27). The exact mechanism of elevation of this message by ischemia is not yet known. It is possible that there are either increases in transcription, decreased mRNA degradation, or a combination of both. Acidosis can act as a specific mechanism to increase message and protein levels through both increased transcription and decreased mRNA degradation. This occurs in the cases of phosphoenolpyruvate carboxykinase and Na^+/HCO_3^- cotransporter (33-36). In our earlier study, with shorter perfusion times of the rabbit myocardium, (26) we noted a very slight trend towards increased expression of this message. In the present study longer periods of perfusion were required in order to observe a significant effect, and this was only shown in low-flow ischemia. Neither more severe low-flow ischemia (1 ml/minute) nor global ischemia (0 ml/minute) resulted in increased Na+/H+ exchanger message. On the contrary, there was a slight decline in the amount of message both in absolute terms or relative to glyceraldehyde 3-phosphate dehydrogenase (Figures 2-2 and 2-3). The reason for this cannot be stated with certainty. However, the results of the contractile function measurements clearly show that the longer severe ischemic insults produced severely damaged tissue. It is likely that Na+/H+ exchanger message is only increased in tissue which is not severely damaged. Possibly, beyond a certain point, the ischemia is so severe that the cellular mechanisms responsible for increased message expression no longer function. It is interesting, however, that even after 3 hours of complete global ischemia there was still intact message for both Na+/H+ exchanger and glyceraldehyde 3-phosphate dehydrogenase (Figure 2-2).

Though we did not measure intracellular pH in the perfused hearts, we showed significant functional changes in hearts perfused at 3 ml/minute or less. These effects may be indicative of the effect of intracellular acidosis which is known to cause decreased contractility (1-3). It should be noted that perfusion of rat hearts at low flow rates (2 ml/min) has been shown to cause decreases in intracellular pH in the myocardium (37). In addition perfusion of ferret hearts under decreased coronary pressure has a similar effect (38). However, Weiss *et al.* (37) used somewhat larger rats in their study and flow rates of 5 ml/min did not reduce intracellular pH. It would thus appear that in the present study the reduction in flow rate to 3 ml/min is approximately borderline for reducing intracellular pH. We did however, note a large reduction in contractility in these hearts (Figure 2-1). This suggests that significant physiological effects occured in the hearts perfused at 3 ml/minute. However, further experiments are necessary to prove decisively that intracellular pH declined in these tissues.

Though we found an increase in the level of mRNA for the Na+/H+ exchanger in the hearts perfused at 3 ml/minute, we did not think that significant protein synthesis would occur within this time. To examine the possibility that acidosis could upregulate Na+/H+ exchanger activity we used a closely related model that could be treated for a longer time. Primary cultures of isolated myocytes were made and treated with chronic low external pH. The results showed that chronic low external pH can increase the activity of the Na+/H+ exchanger protein in intact myocardial cells. This more directly shows that acidosis can cause an increase in Na+/H+ exchanger activity. The results are similar to those shown in renal tissues (22-26). It is thus apparent that the mammalian myocardium has a mechanism to increase the level of message and activity of the Na+/H+ exchanger, at least in the examples of the experimental models used in the present study. It should be noted that the 2 models used, isolated myocytes and the isolated perfused heart, both differ significantly from each other and from the intact mammalian myocardium. The mechanisms involved in these 2 models could differ from one another. The mechanisms of increased activity in the isolated myocytes might not involve effects on Na+/H+ exchanger message and protein levels, and could be an effect on regulation of the protein. However, since acidosis has been shown to increase message and protein levels in other tissues (22-26) the most likely explanation is one involving the same mechanism in isolated myocytes.

Overall, our results suggest that in the mammalian myocardium, ischemia and acidosis can cause increases in the amiloride sensitive Na+/H+ exchanger (NHE1) message and activity. Whether ischemia in the intact animal can lead to increased message and activity remains to be proven. The Na+/H+ exchanger has been shown to be an important contributing factor during production of cell necrosis and arrhythmia associated with reperfusion. Therefore, it is possible that an increased production of this message during chronic ischemic episodes such as angina could lead to increased incidence of arrhythmias and cell necrosis. Future studies may examine this possibility.

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CHAPTER THREE

Activation of the Na+/H+ Exchanger Gene by the Transcription Factor AP-2

A version of this chapter also appears in The Journal of Biological Chemistry (1995),270(3);1375-1381.

Jason R. B. Dyck, Norma Lucena C.L. Silva and Larry Fliegel.

INTRODUCTION

The Na^+/H^+ exchanger is a mammalian plasma membrane protein that mediates the exchange of intracellular protons for extracellular sodium. It is involved in pH regulation (1), control of cell volume (2), and is stimulated by growth factors (3). Several different isoforms of the protein exist which have been designated NHE1 to NHE4. The NHE1 isoform is the widely distributed 'housekeeping' isoform of the family present in most if not all mammalian cells (4). Although the mechanism of regulation of protein levels is extremely important, only a few studies have examined the human NHE1 gene (5, 6). Miller *et al.* (5) demonstrate that the 5' flanking region of the human NHE1 gene contains a number of putative sites for DNA binding transcription factors, any of which could regulate the exchanger gene transcription. Of these sites, only AP-1 has been implicated to have a possible role in activating the NHE1 gene (7). More recently, a more in-depth study of the human NHE1 gene has analyzed the proximal regulatory elements of the promoter (6). To date, however, there has been no direct identification of any specific transcription factor responsible for regulating the NHE1 promoter.

Expression of the message and activity of the Na^+/H^+ exchanger has been shown to vary greatly depending on the state of differentiation (8) and a variety of other stimuli (9-11). The regulation of the NHE1 gene, however, involves the link between DNA-binding protein motifs located in promoter and enhancer sequences and the general transcription machinery. Various cis-elements of the gene may be able to activate transcription through responses to certain extracellular messages. One trans-element involved in transcriptional regulation is the transcription factor AP-2. Transcriptional activation by AP-2 involves the 52-kD AP-2 protein binding to a specific DNA motif found in the cis-regulatory region of the gene (12). AP-2 activity is regulated in a cell-type specific manner (13) and is induced by phorbol esters, retinoic acid and cAMP (12-14). Also, mRNA levels of AP-2 have been shown to increase dramatically upon differentiation indicating that the expression of AP-2 is regulated during differentiation (14).

In this study we demonstrate, for the first time, a specific region within the Na^+/H^+ exchanger gene that is involved in regulating transcription. Moreover, we have shown that this DNA motif interacts with AP-2 or an AP-2-like transcription factor. We report the sequence of the upstream region of the mouse NHE1 gene, the identification and characterization of an AP-2 site and the importance of this region for NHE1 gene transcription.

EXPERIMENTAL PROCEDURES

Materials

Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim (Laval, Quebec, Canada) and Bethesda Research Laboratories (Gaithersburg, MD, USA). The pBluescript plasmids were from Stratagene (LaJolla, CA, USA). Plasmid pXP-1 was a gift from Dr. M. Nemer of the Institute de recherches cliniques de Montreal, Montreal, Quebec, Canada. pRSVAP2 was a generous gift of Dr. R. Gaynor from U. T. Southwestern Medical School, Dallas, Texas, USA. Hybond-N+ nylon membranes were obtained from Amersham (Oakville, Ontario, Canada). The Gel Shift Assay System was purchased from Promega (Madison, Wisconsin, USA). $[\gamma^{32}P]$-dCTP,

[α-^{32}P]-dATP, [γ-^{32}P]-dATP and [α-^{35}-S]-dATP were from ICN. All other chemicals were of analytical or molecular biology grade and were purchased from Fisher Scientific (Ottawa, Ontario, Canada), Sigma (St. Louis, MO, USA) or BDH (Toronto, Ontario, Canada).

Screening and Sequencing of Genomic Clones

Screening of a mouse lambda Gem-11, genomic DNA library (Promega, Madison, Wisconsin, USA) was with a fragment of the rabbit cardiac Na^+/H^+ exchanger (NHE1) cDNA clone (1-691 bp, 15) and a fragment of the 5' untranslated and coding region (1-900) of the human Na^+/H^+ exchanger as described earlier (16). Filters were routinely washed with 1 x SSC at 42°C. This resulted in the isolation of two genomic DNA clones that were excised by digestion with Sfi-1. Fragments of each clone were subcloned into pBluescript (KS-). For sequencing reactions a progressive series of deletions was constructed with exonuclease III in both directions. Double stranded DNA sequencing of fragments and plasmids was by the DNA Sequencing Lab in the Department of Biochemistry, using an Applied Biosystems, Model 373A DNA sequencer. Synthetic oligonucleotides were made at the same location using an Applied Biosystems, Model 392 DNA/RNA synthesizer.

Primer Extension

Primer extension analysis was with a 20 bp oligonucleotide of the sequence 5' TCC TTC GGT CAG CTC CAG CT 3'. The primer was endlabeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase to a specific activity of 1-3 x 10^9 cpm/μg. Unincorporated [γ-^{32}P]-ATP was removed by purification with a Sephadex G-50 column. One pmol of primer was hybridized to 15 μg of mouse kidney poly $(A)^+$ RNA in a primer extension solution containing 50 mM Tris-

HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP, 0.5 mM spermidine. Mixtures were annealed at 58 °C X 20 minutes and then at room temperature for 10 minutes. The sample was added to a reverse transcription buffer containing the same solution plus 2.8 mM sodium pyrophosphate and 8 units of AMV reverse transcriptase. After 30 minutes at 42°C the reaction was terminated by the addition of loading dye and the samples were analyzed on a 7% polyacrylamide/urea gel.

Cell Culture

NIH 3T3 cells were obtained from Dr. J. Stone of the Department of Biochemistry, University of Alberta. They were maintained in DMEM media supplemented with 10% fetal bovine serum. Hep G2 cells were provided by Dr. Z. Yao of the Department of Biochemistry, University of Alberta. These cells were propagated in DMEM media with 2 mM L-glutamine 10% FCS, 100 Units/ml penicillin, 100 mg/ml streptomycin and a pH of 7.2.

Reporter Plasmid Construction

pXP-1MP was constructed by digesting a 2.2 kb fragment containing the promoter/enhancer region with Pst 1 and Sma 1. An intermediate plasmid that contained a Pst 1 site flanked by a Hind III and Sal 1 was used to transfer the Pst-Sma 1.1 kb fragment into the Hind III and Sal 1 site of pXP-1. The 1.1 kb fragment contains base pairs -1085 to +22 of NHE1. To construct pMP+AP2 plasmid, two oligonucleotides were used as primers for polymerase chain reaction (PCR; # 1, ttgg atc CGT GAC ACT TCC TTC CCT and #3, cc ttc gaa GGG TCC CGC GGT AGC GGA) were synthesized to amplify base pairs -125 to +22 of the gene. The PCR product had the restriction enzyme sites Bam HI and Sma 1 generated on either end and was inserted directionally into pXP-1.

Similarly, pMP-AP2 plasmid was made using the product of the primers # 3 and #2 (#2, ttgg atc CTG CAC CGC GCG GGC GCT) and the 114 bp product (-92 to +22) was inserted into pXP-1. Mutations in pMP(MUT)AP2 were generated by PCR, using a mismatched oligonucleotide (#1-mut, ttgg atc CGT GAC ACT TCC TTT TTT AAG TAA TAT AAG CCG CTG CAC CG) similar to oligonucleotide #1 above. Other mutations in the AP-2 containing region were made using a similar procedure. pRSV-AP2 was constructed by digesting pRSVAP2 with Eco RI and removing the AP2 gene. All plasmids were sequenced to verify proper orientation and fidelity of PCR.

Transfection and Reporter Assays

Cells were plated onto 35 mm dishes. Each dish received 5 μg of luciferase reporter plasmid and 5 μg of pSV-β-Galactosidase plasmid as an internal control. In some cases (i.e., Hep G2 cells) 5 μg of a third plasmid was also cotransfected (i.e., pSV±AP2). Both NIH 3T3 cells and Hep G2 cells were transiently transfected using the $CaPO_4$ precipitation technique (17). After 36 hours cells were harvested and the cell lysate was assayed for both luciferase activity and β-Galactosidase activity. Each luciferase assay contained 30 ml of the cell lysate and 100 ml of the luciferase assay reagent (Tricine 20 mM, Magnesium Carbonate 1.07 mM, $MgSO_4$ 2.67 mM, EDTA 0.1 mM, DTT 33.3 mM, luciferin 470 mM, ATP 530 mM, Coenzyme A 270 mM, and BSA 1 ng/ml). The β-Galactosidase assay included 5 μl of cell lysate, 95 μl H_20 and 20 μl of o-Nitrophenyl-ß-D-galactopyranoside, incubated at 37^oC for 60 minutes. Luciferase activity was assayed with an LKB luminometer and normalized to β-Galactosidase for efficiency of transfection. Results are reported as Mean ± SE; where not shown the SE was too small to be displayed.

DNase I Footprinting

The 206 bp probe for DNase I footprinting was isolated from pXP-1MP using the restriction enzymes Rsa I and Hind III. The DNA probe was gel purified and was radiolabeled by filling in the protruding 5' end generated by the Hind III. The reaction mixture contained dATP, dGTP, dTTP, [γ-^{32}P]-dCTP, and the Klenow fragment of *E. coli* DNA polymerase I. The fragment contained the mouse NHE1 promoter region -171 to + 22 along with 13 bp of the 5' end of the pXP-1 plasmid. Five ng of DNA (30 000 cpm) was incubated with 50 µg of NIH 3T3 nuclear extracts at 4°C overnight and then treated with DNase I at room temperature for 10 seconds to 2 min. It was terminated with 2.5 µl of stop solution (125 mM Tris-HCl (pH 8.0), 125 mM EDTA, 3% SDS) and proteins removed by phenol/chloroform extraction. After precipitation the sample was resuspended in 98% formamide-dye, and electrophoresed on a 6% acrylamide/ 7M urea sequencing gel.

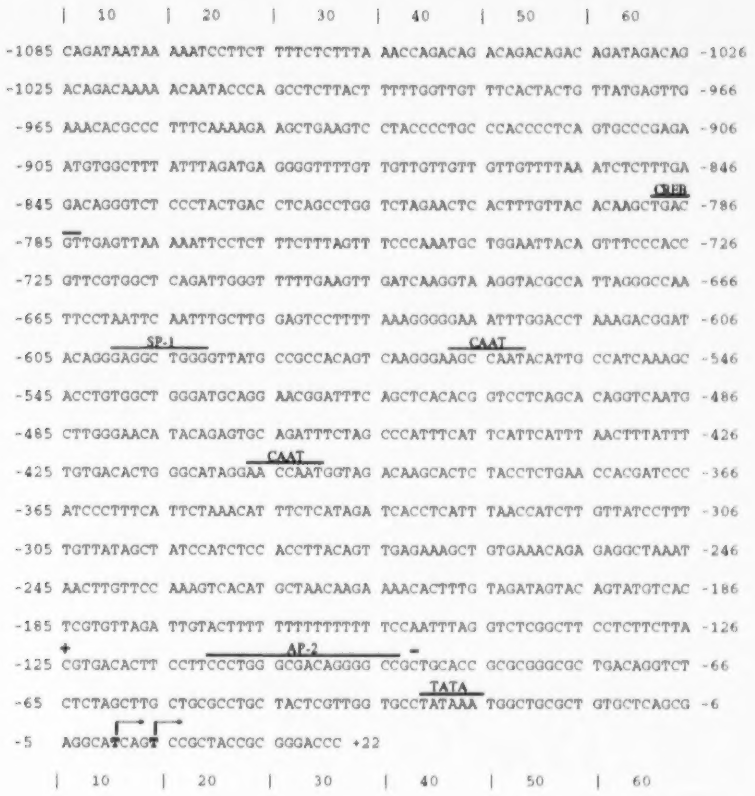
DNA Binding Assays

Nuclear extracts were prepared from NIH 3T3 cells according to the procedure of Schreiber *et al.* (18). The synthetic oligonucleotides of the sequence 5' TTC CTT CCC TGG GCG ACA GGG GCC 3' (MPAP2a) and 5' GGC CCC TGT CGC CCA GGG AAG GAA 3' (MPAP2b) were made corresponding to the mouse promoter AP-2 region. Alternatively, the mutant oliogonucleotides of the sequence 5' TTC CTT TTT TAA GTA ATA TAA GCC 3' (MUTAP2a) and 5' GGC TTA TAT TAC TTA AAA AAG GAA 3' (MUTAP2b) were also used for analysis. The oligonucleotides were end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. They were heated to 95°C for 2 minutes and cooled to room temperature overnight for annealing. DNA binding reactions were for 10 minutes at room temperature and contained: 30 000 CPM of [^{32}P]-labeled oligonucleotides was mixed with purified AP-2 (1.4 mg) or NIH 3T3 nuclear extract (5μg) in a binding buffer (4% glycerol, 1.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5 and 0.05 μg/ml poly dI:dC). After electrophoresis on 4% polyacrylamide gels. Gels were dried and exposed to X-ray film for 16 hours. The non-competing oligonucleotide, SP-1, was: 5' ATT CGA TCG GGG CGG GGC GAG C 3'. In some experiments nuclear extracts were treated prior to band shift to specifically remove AP-2 protein before gel mobility shift assay. 20 ul of a 10% solution of Protein-A was incubated with 2.5 μg of AP-2 antibody (IgG, Santa Cruz Biotechnology Inc.) for 1 hr at 4°C. After 1 hour the solution was washed 3 times (20mM tris, pH 7.9, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, and 1mM PMSF), and mixed with 5 μl of NIH 3T3 nuclear extracts and incubated at 4°C for 16 hours. The mixture was then spun at 6500 rpm for 10 min. and the supernatants were collected. Control experiments contained equal amounts of irrelevant IgG.

To examine if the Na^+/H^+ exchanger gene could directly bind purified AP-2 protein we isolated and end labeled a DNA fragment of the gene from bp -171 to +22 of the promoter. This fragment contained the putative AP-2 binding site and the surrounding nucleotides. Gel mobility shift assay was performed as described above.

RESULTS

Two positive clones were obtained by library screening with probes from both the 5'UTR and the coding region of the human and rabbit NHE1 gene. Clone 3-1 contained most of the coding region along with the entire 3' untranslated region and clone 3-3 included a section of the coding region, the 5' untranslated region and approximately 10 kb upstream from the 5' untranslated region. A 2.2 kb fragment from clone 3-3 was sequenced in both directions using exonuclease digestions. The deduced sequence of the first 40 amino acids of the mouse 5' coding region was compared with other Na^+/H^+ exchangers (NHE 1-4) from a variety of species. It was 90% to 83% identical to the other NHE1 isoforms with the hamster showing the greatest identity. No significant identity was seen with the other isoforms of the exchanger (NHE 2 - NHE 4, not shown). The sequence of the mouse promoter/enhancer region is shown in Figure 3-1. It contains putative recognition sites for several transcription factors. This includes a TATA box, two CAAT boxes, an SP-1 site, a CREB site and an AP-2 site (Figure 3-1). The sequence TATAAA in the mouse NHE1 gene is identical to the well characterized consensus sequence for the mammalian TATA box. Two CAAT boxes are present at sites -407 to -401 and -568 to -562 along with an SP-1 site at position -600 to -592. Both CAAT and SP-1 are characteristic binding sites for transcriptional regulatory proteins seen in many eukaryotic promoters (19, 20). The CREB-site, at position -789 to -784, is a putative cAMP response element that can activate transcription upon cAMP or Ca^{2+} stimulation (21). The site may be of significance since it has been shown that cAMP can modulate exchanger activity acutely (22). Finally, a putative binding site for one of the more well known transcription factors, AP-2 is located -111 to -94 and was also a candidate for transcriptional regulation .



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      |  10      |  20      |  30      |  40      |  50      |  60

-1085 CAGATAATAA AAATCCTTCT TTTCTCTTTA AACCAGACAG ACAGACAGAC AGATAGACAG -1026
-1025 ACAGACAAAA ACAATACCCA GCCTCTTACT TTTTGGTTGT TTCACTACTG TTATGAGTTG -966
 -965 AAACACGCCC TTTCAAAAGA AGCTGAAGTC CTACCCCTGC CCACCCCTCA GTGCCCGAGA -906
 -905 ATGTGGCTTT ATTTAGATGA GGGGTTTTGT TGTTGTTGTT GTTGTTTTAA ATCTCTTTGA -846
                                                                CREB
 -845 GACAGGGTCT CCCTACTGAC CTCAGCCTGG TCTAGAACTC ACTTTGTTAC ACAAGCTGAC -786
 -785 GTTGAGTTAA AAATTCCTCT TTCTTTAGTT TCCCAAATGC TGGAATTACA GTTTCCCACC -726
 -725 GTTCGTGGCT CAGATTGGGT TTTTGAAGTT GATCAAGGTA AGGTACGCCA TTAGGGCCAA -666
 -665 TTCCTAATTC AATTTGCTTG GAGTCCTTTT AAAGGGGGAA ATTTGGACCT AAAGACGGAT -606
              SP-1                                 CAAT
 -605 ACAGGGAGGC TGGGGTTATG CCGCCACAGT CAAGGGAAGC CAATACATTG CCATCAAAGC -546
 -545 ACCTGTGGCT GGGATGCAGG AACGGATTTC AGCTCACACG GTCCTCAGCA CAGGTCAATG -486
 -485 CTTGGGAACA TACAGAGTGC AGATTTCTAG CCCATTTCAT TCATTCATTT AACTTTATTT -426
                         CAAT
 -425 TGTGACACTG GGCATAGGAA CCAATGGTAG ACAAGCACTC TACCTCTGAA CCACGATCCC -366
 -365 ATCCCTTTCA TTCTAAACAT TTCTCATAGA TCACCTCATT TAACCATCTT GTTATCCTTT -306
 -305 TGTTATAGCT ATCCATCTCC ACCTTACAGT TGAGAAAGCT GTGAAACAGA GAGGCTAAAT -246
 -245 AACTTGTTCC AAAGTCACAT GCTAACAAGA AAACACTTTG TAGATAGTAC AGTATGTCAC -186
 -185 TCGTGTTAGA TTGTACTTTT TTTTTTTTTT TCCAATTTAG GTCTCGGCTT CCTCTTCTTA -126
      +                         AP-2               -
 -125 CGTGACACTT CCTTCCCTGG GCGACAGGGG CCGCTGCACC GCGCGGGCGC TGACAGGTCT -66
                                              TATA
  -65 CTCTAGCTTG CTGCGCCTGC TACTCGTTGG TGCCTATAAA TGGCTGCGCT GTGCTCAGCG -6
   -5 AGGCATCAGT CCGCTACCGC GGGACCC +22

      |  10      |  20      |  30      |  40      |  50      |  60
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Figure 3-1. **Nucleotide sequence of the promoter/enhancer region of the mouse NHE1 isoform.** Nucleotides for the coding strand are numbered beginning with the first start site of transcription, shown as +1. Start sites for transcription are denoted with arrows at positions +1 and +5. Horizontal lines indicate putative binding sites for DNA-binding proteins, SP-1, CAAT, CREB, TATA, and AP-2. The first base pairs of the plasmids pMP+AP2 and pMP-AP2 are indicated by the + and - respectively.

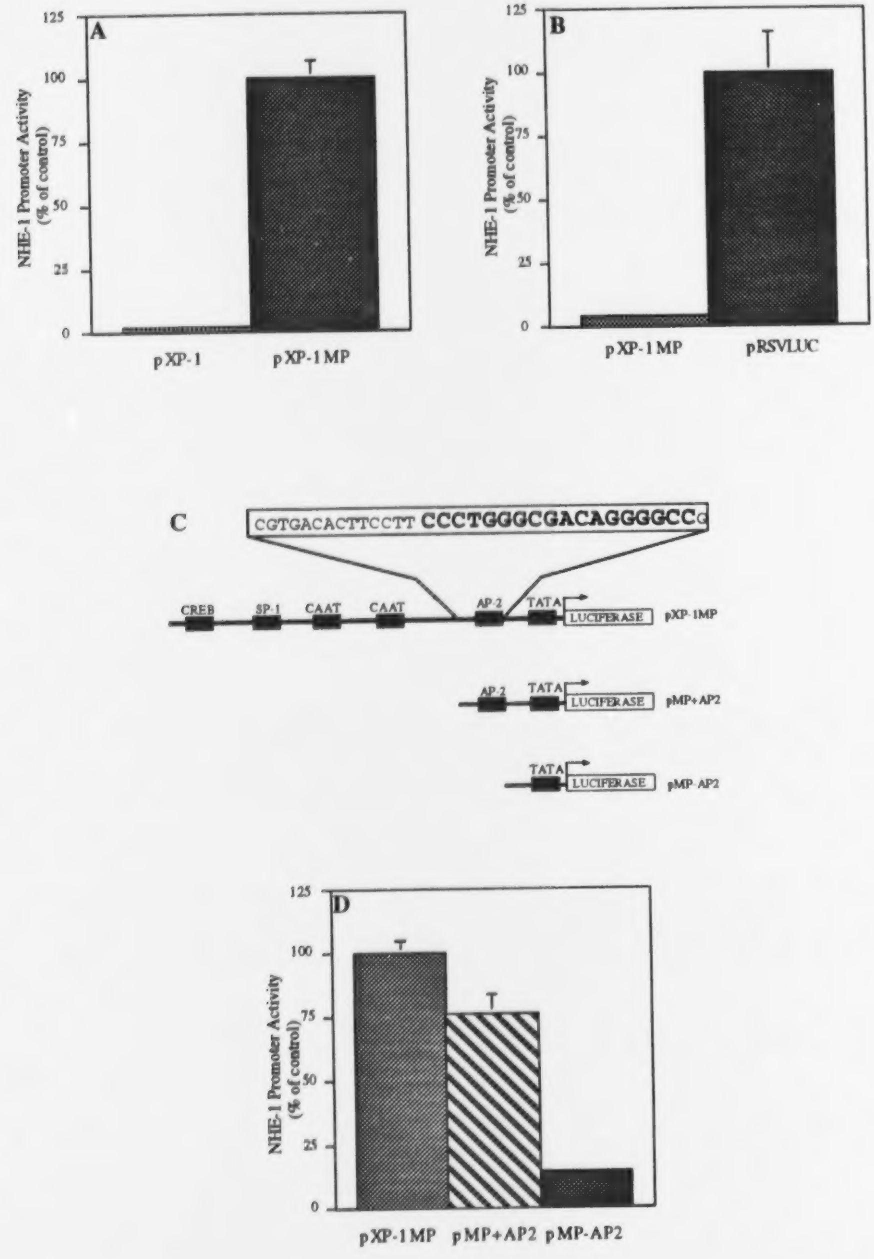
The start sites for transcription were demonstrated to occur at base pairs +1 and +5 (Figure 3-1) and occur at nucleotides that are 26 and 30 bp from the TATA box. The two start sites and their distance from the TATA box are identical to that reported for the human promoter (5). Our initial experiments tested whether a 1.1 kb sequence upstream from the start site was indeed a promoter, and if so its relative ability to direct transcription in mouse NIH 3T3 cells. When the level of luciferase activity from pXP-1MP was compared to pXP-1, a 73-fold increase in activity was seen (Figure 3-2A). Transfection experiments also (Figure 3-2B) showed that pXP-1MP induced 23.8-fold less luciferase activity than the Rous Sarcoma Virus RSV promoter located 5' to the luciferase gene (pRSVLUC). This indicates that the promoter for the mouse NHE1 gene is relatively weak at directing transcription in comparison to pRSVLUC. These results could account for the low levels of exchanger protein and mRNA present in mammalian tissues (4). Another set of experiments examined the relative ability of an 8 kb fragment of the NHE1 promoter/enhancer region to direct transcription. This fragment did not stimulate transcription greater than the 1.1 kb fragment (not shown).

To examine which of the identified consensus sequences are involved in regulation of NHE1 transcription we constructed several different plasmids containing varying fragments of the promoter/enhancer region. We initially removed all base pairs upstream of the AP-2 site from pXP-1MP. This construct was named pMP+AP2. The second construct, pMP-AP2, was identical to pMP+AP2 except for the further deletion of the AP-2 site. We then examined the ability of these two plasmids to direct transcription of the luciferase gene. They were transfected into NIH 3T3 cells along with pSV-ß-Galactosidase which was used to normalize for any differences in transfection efficiency.

Figures 3-2C and 3-2D are comparisons between the plasmid containing the entire sequenced region (pXP-1MP), the plasmid containing the AP-2 binding site (pMP+AP2) and the plasmid with the AP-2 binding site removed (pMP-AP2). Deleting all the putative transcription factor binding sites, except AP-2 caused some decrease in NHE1 transcription (Figure 3-2D). Removal of the AP-2 containing region decreased transcription 6-fold (Figure 3-2D).

To confirm the importance of the 33 bp deleted region we introduced several mutations into the AP-2 consensus sequence of the pMP+AP2 construct. The substitutions are shown in Figure 3-3A. When the mutant construct #1 (pMP(MUT)AP2) is transfected into NIH 3T3 cells we see a 7.9-fold decrease in the level of luciferase activity (Figure 3-3B) which is comparable to the levels seen when the entire AP-2 region is deleted. To localize the nucleotides involved in this activity two other mutations were generated. Mutation #2 involved a 5 basepair modification of an adjacent region. This had little effect on activity of the promoter. When another two mutations were added to these existing 5 (mutation #3) a small decrease in luciferase activity was seen. We performed DNase I footprinting analysis to determine if the specific protein from NIH 3T3 nuclear extracts could interact with the DNA sequence containing this AP-2 consensus site. Nuclear factors protected a region corresponding to -106 to -95 of the mouse NHE1 promoter region (Figure 3-4). This region lies within the AP-2 consensus site which is located in the 33 bp fragment of DNA.

Figure 3-2. **NHE1 promoter activity in mouse NIH 3T3 fibroblasts.** A, mouse fibroblasts were transiently transfected with either a 1.1 Kb fragment of the mouse Na^+/H^+ exchanger (NHE1) promoter/enhancer region linked to a luciferase reporter gene (pXP-1MP) or with the reporter plasmid without the 1.1 Kb insert (pXP-1). B, fibroblasts were transfected with either pXP-1MP as in (A) or with pRSVLUC. C, fragments of the promoter were inserted upstream of luciferase gene in the vector pXP-1. pXP-1MP contains from -1085 to +22 of the mouse promoter/enhancer region. pMP+AP2 contains the region between -125 and +22 and pMP-AP2 contains the region between -92 and +22. The boxed sequence indicates the region containing the putative AP-2 site that is deleted in pMP-AP2 and the bold letters denote the putative AP-2 site. D, mouse fibroblasts were transiently transfected with either pXP-1MP, pMP+AP2 or pMP-AP2. For all transfection experiments the results reported were obtained from at least three independent experiments each carried out in triplicate using at least two different DNA preparations for each plasmid. pSV-ß-Galactosidase was used as an internal control.


A
NHE-1 Promoter Activity (% of control)
125
100
75
50
25
0
pXP-1
pXP-1MP
B
NHE-1 Promoter Activity (% of control)
125
100
75
50
25
0
pXP-1MP
pRSVLUC
C
CGTGACACTTCCTT CCCTGGGCGACAGGGGCCG
CREB
SP-1
CAAT
CAAT
AP-2
TATA
LUCIFERASE
pXP-1MP
AP-2
TATA
LUCIFERASE
pMP+AP2
TATA
LUCIFERASE
pMP-AP2
D
NHE-1 Promoter Activity (% of control)
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pMP+AP2
pMP-AP2

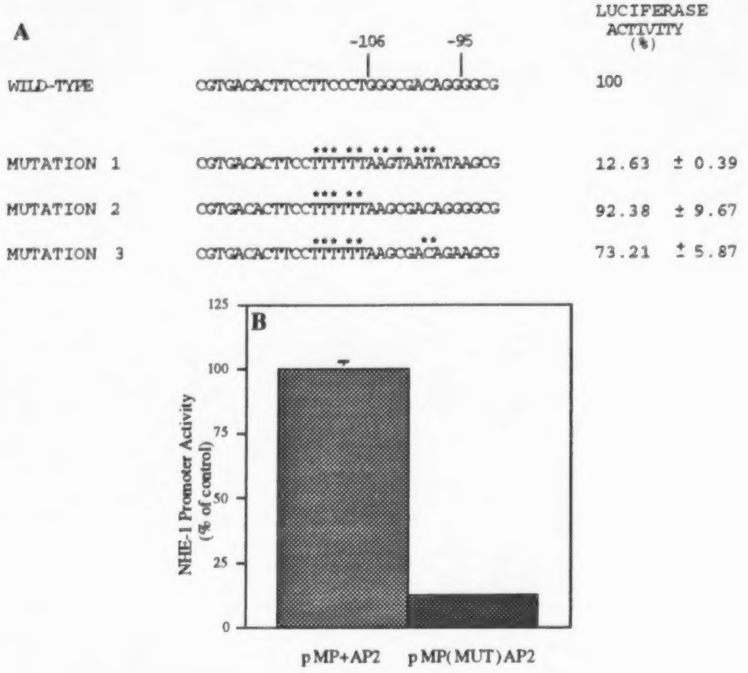


Figure 3-3. **Effect of mutant constructs on NHE1 promoter activity in NIH 3T3 cells.** A, comparison of the wild-type and 3 mutant constructs of the 33 bp sequence containing the AP-2 site. All the mutations are indicated by asterisks and the numbers correspond to the positions of the nucleotides relative to the first transcription initiation site. The numbers also represent the region of DNA which is protected by AP-2 protein from nuclear extracts of NIH 3T3 cells during DNase I footprint analysis. The level of luciferase activity in NIH 3T3 cells for the wild-type and the mutations are shown to the right and are expressed as % of the pMP+AP2 construct. B, mouse fibroblasts were transiently transfected with either pMP+AP2 or pMP(MUT)AP2. Harvest and transfections were as described for Figure 4-2.

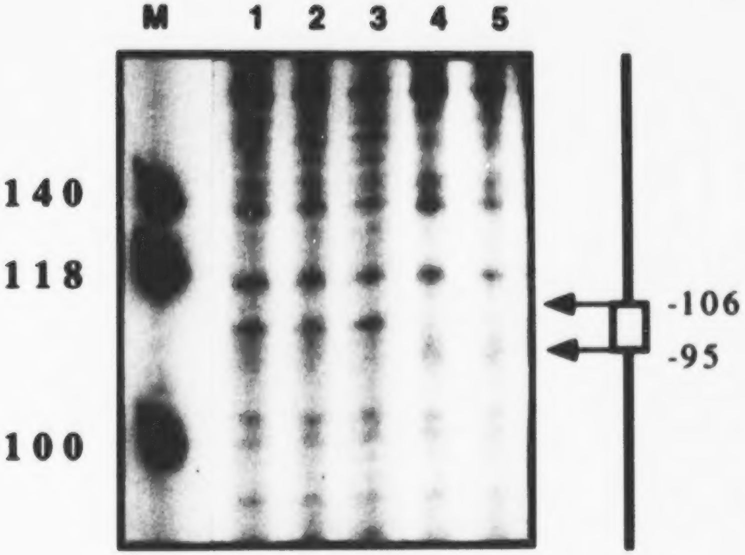


Figure. 3-4. **DNase I footprinting analysis of the mouse NHE1 promoter**. DNase I footprint analysis was performed with the -171 to + 22 fragment of the NHE1 promoter (lanes 1-4). The naked fragment of DNA was treated with DNase I (0.1 Units / lane 1 and 0.2 Units / lane 2) for 10 seconds at room temperature. The fragment of DNA was also treated with DNase I (1.0 Units/lane 3 and 2.0 Units/lane 4) for 2 min. at room temperature after incubation with nuclear extracts from NIH 3T3 cells. Lane M represents the [γ-^{32}P]-dATP end labeled molecular weight markers whose lengths are shown to the left. The protected region of DNA is indicated to the right as an open box, and the numbers correspond to the position of the nucleotides in the DNA sequence.

NIH 3T3 cells have one of the most abundant AP-2 levels of a number of murine cell lines and tissues (13, 23). Hep G2 are human liver hepatoma cells which have been shown to be deficient in AP-2 mRNA and have little, if any, AP-2 binding and transcription activity (14). To confirm that AP-2 protein or an AP-2-like protein regulates transcription of the mouse NHE1 gene Hep G2 cells were transfected with pMP+AP2 and cotransfected with either a plasmid expressing AP-2 protein (pRSVAP2) or the identical plasmid that cannot express AP-2 protein (pRSV-AP2). The results (Figure 3-5) show that cotransfection with an AP-2 expression plasmid causes a large 2-fold increase in the activity of the NHE1 promoter. When the mutated construct pMP(MUT)AP2 is transfected into Hep G2 cells along with pRSVAP2 there is no increase in activity of the mouse NHE1 promoter as compared to the wild-type containing plasmid (Figure 3-5).

To investigate the role of AP-2 protein as an enhancer of NHE1 transcription in more detail, we examined the ability of the AP-2 motif to bind AP-2 protein. A short double stranded oligonucleotide (MPAP2a,b) of 24 base pairs was synthesized, that consisted of the sequence 5' TTC CTT CCC TGG GCG ACA GGG GCC 3'. Gel retardation assays showed that this oligonucleotide bound to the purified AP-2 protein (Figure 3-6A, lane 1). Competition experiments were done to show specific binding of AP-2, using unlabelled MPAP2a,b oligonucleotide as the competitor and an unlabelled SP-1 oligonucleotide as a non-competitor. Competitor concentrations of 6x, 12x and 50x blocked the protein-DNA interaction while 100x excess of non-competitor had no effect (Figure 3-6A, lanes 2-5 respectively). To further characterize the protein-binding ability of the mouse AP-2 consensus site, nuclear extracts from NIH 3T3 cells were used.

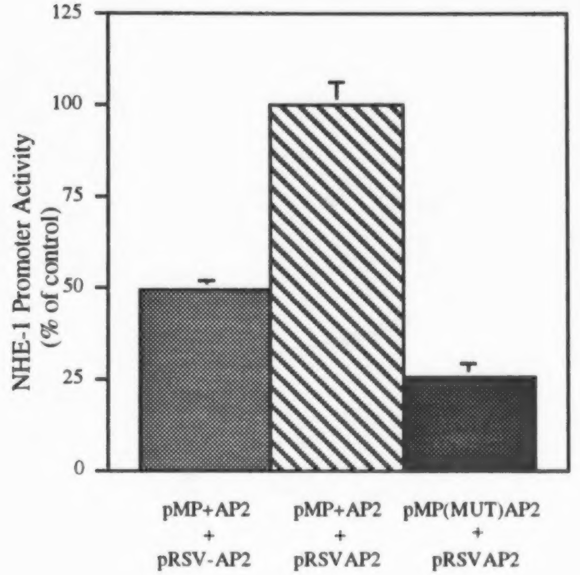


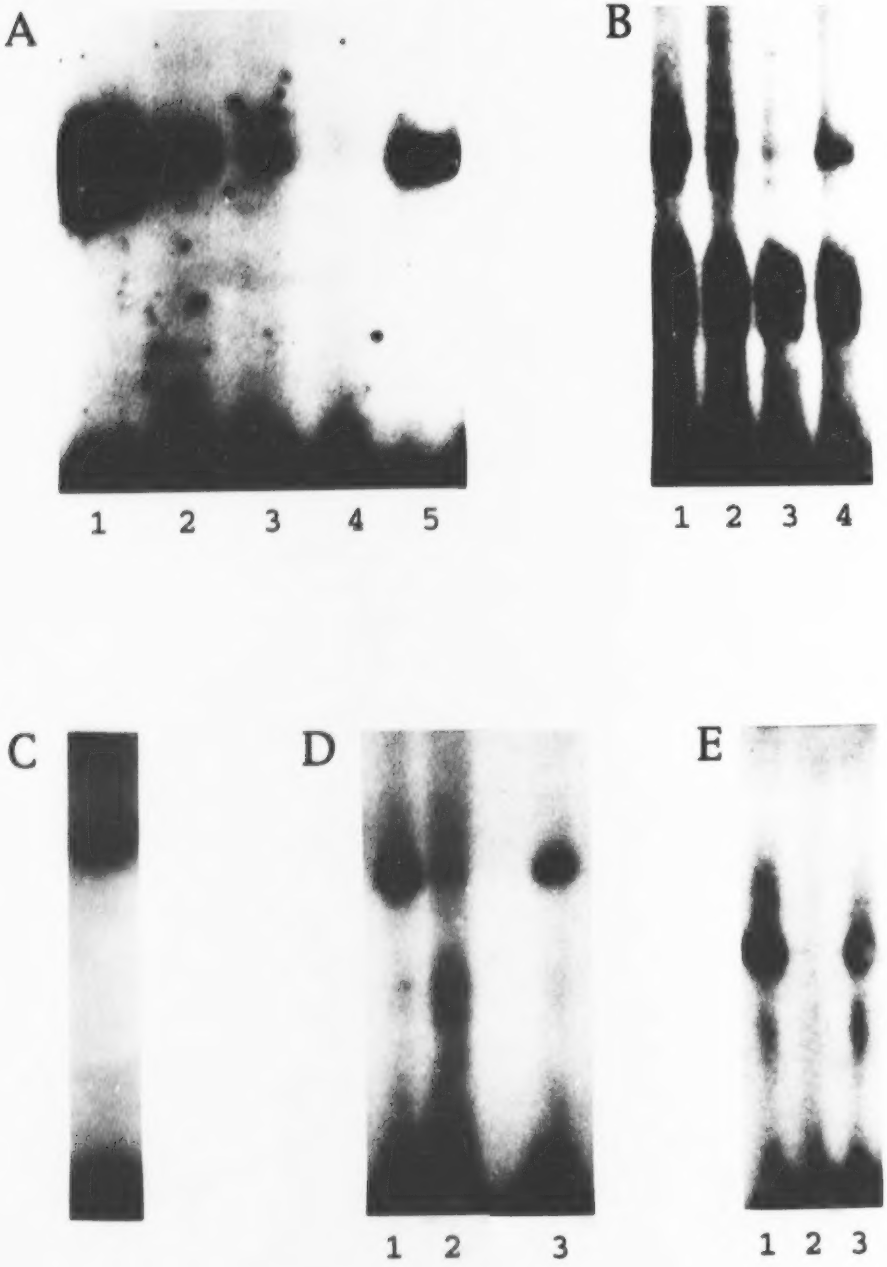
Figure 3-5. **Effects of AP-2 on NHE1 promoter activity in human Hep G2 cells.** Human liver hepatoma cells (Hep G2) were transiently transfected with either pMP+AP2 or pMP(MUT)AP2 and cotransfected with either an AP-2 expression plasmid (pRSVAP2) or the same expression plasmid with the AP-2 gene deleted (pRSV-AP2) as indicated. Harvest and transfections were as described for Figure 4-2.

The results with purified AP-2 protein were similar to those seen in experiments with nuclear extracts. Nuclear extracts of NIH 3T3 cells bound to the AP-2 containing synthetic oligonucleotide (MPAP2a,b) and 12x and 25x excess unlabelled competitor oligonucleotide decreased the binding (Figure 3-6B lanes 2 and 3), while 100x excess of non-competitor did not (lane 4). Some binding of smaller size was apparent, but competition experiments revealed that the interaction was non-specific (lanes 2 and 3). We also examined whether a 193 bp fragment cut directly from the promoter could bind purified AP-2 protein. The results are shown in Figure 3-6C. An intense band was seen indicative of AP-2 binding to the labeled fragment of the gene. In the absence of the protein the band was not apparent. The synthetic oligonucleotides containing the sequence for the mutated mouse putative AP-2 binding site (MUTAP2a,b) were used in another bandshift assay.

The oligonucleotides MUTAP2a,b only bound small amounts of protein from nuclear extracts of NIH 3T3 cells (Figure 3-6D lane 2) in comparison to MPAP2a,b (Figure 3-6D lane 1). Also, the unlabelled mutated oligonucleotides were unable to compete with MPAP2a,b confirming their reduced ability to bind to the protein. Finally AP-2 was specifically removed from the nuclear extract by immunoprecipitation with anti-AP-2 antibody. This resulted in greatly reduced binding to MPAP2a,b by the nuclear extracts (Figure 3-6E).

The AP-2-like site located in the NHE1 gene varies slightly from other known AP-2 consensus sequences (Figure 3-7 A). We have, however, shown purified AP-2 protein to bind to this region, thus strongly suggesting that AP-2 can indeed bind to this aberrant site. Although AP-2 is the most likely candidate for binding to, and regulating the NHE1 gene it is also possible that

Figure 3-6. **DNA-mobility shift binding assay and competition analysis of the mouse NHE1 AP-2 site.** The labeled 24 base pair oligonucleotide MPAP2a,b (positions -117 to -94) was incubated with purified human AP-2 protein or nuclear extracts for 10 minutes at room temperature. The binding mixtures were analyzed by electrophoresis on 4% polyacrylamide gels as described under "Experimental Procedures". A, lane 1, purified AP-2 protein (1.4 μg) alone added to the binding reactions; lanes 2-4, competitions with 6-fold, 12-fold and 50-fold excess of unlabelled MPAP2a,b respectively; lane 5, competition with 100-fold excess of a nonspecific sequence competitor, SP-1. B, nuclear extracts from NIH 3T3 were used for mobility shift binding assay with MPAP2a,b and 5 mg of NIH 3T3 cell nuclear extract as described in (A). lane 1, nuclear extract alone added to the binding reactions; lanes 2 and 3, competitions with 12-fold and 25-fold unlabelled MPAP2a,b, respectively, and lane 4 shows competition with 100-fold excess of a nonspecific sequence competitor SP-1. C, A fragment of Na^+/H^+ exchanger genomic DNA containing the AP-2 consensus sequence was isolated and labeled with [γ-^{32}P]-ATP as described in "Experimental Procedures". Purified AP-2 protein was incubated with the fragment and the results were analyzed as described in (A). D, the labeled 24 base pair oligonucleotide MUTAP2a,b or MPAP2a,b (positions -117 to -94) were incubated with 5 μg of NIH 3T3 nuclear extract and analyzed as above. Lane 1 is the wild-type oligonucleotide MPAP2a,b, while lane 2 is the mutant oligonucleotide. Lane 3 is a competition assay containing labeled MPAP2a,b and 25-fold excess of cold MUTAP2a,b. E, DNA mobility shift with MPAP2a,b with nuclear extracts untreated (lane 1) or treated (lane 2) via immunoprecipitation to remove AP-2 transcription factor. Immunoprecipitation data was provided by Dr. Norma Lucena C.L. Silva.


A
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4
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B
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4
C
D
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2
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E
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3

an AP-2-like protein binds to this nucleotide sequence. The fact that purified protein and protein from nuclear extracts result in slight changes in the mobility of the NHE1 AP-2 synthetic oligonucleotide, lends some support to the notion of an AP-2-like protein (Figure 3-7B). This variation in mobility, however, may be explained by the simple fact that nuclear extracts contain many proteins which may also have some affinity to this region of the mouse NHE1 gene. Further experiments are needed to fully understand this inconsistency.

A

AP-2 RECOGNITION SITE: $\frac{\mathrm{T}}{\mathrm{C}}\,\mathrm{C}\,\frac{\mathrm{C}}{\mathrm{G}}\,\mathrm{C}\,\mathrm{C}\,\substack{\mathrm{A}\\ \mathrm{C}}\,\mathrm{N}\,\frac{\mathrm{GCG}}{\mathrm{CGC}}$

AP-2 BINDING SITES:

CCCTGGGCGACAGGGGCC	NHE1 (mouse)
TGTGGAAAGTCCCCAGGCTCCCCA	SV40
GGGAACTGACCGCCCGCGGCCCGT	hMT-II_A (human metallothionein IIa)
CCGGGTGTTTCGCCTGGAGCCGCAA	hMT-II_A
GGTGCCCTCTGGCCGCAGGCCAT	hGH (human growth hormone)
CCCTAGTACGGGGGACCGAACAG	hGH
TTAGAGGGCGGGTGGCCGGGAAA	hc-myc (human c-myc)
GGGCGGGACGTGGCGGG	ACC PII (Acetyl-CoA Carboxylase)

B

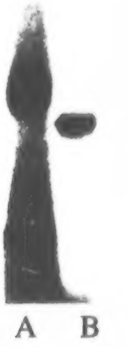

Figure 3-7. **Comparison of AP-2 binding sites and DNA-mobility shift binding assay.** A, the AP-2 recognition site and the different binding sites of AP-2 determined by DNase I footprinting experiments (23). B, mobility shift variation between protein from NIH 3T3 nuclear extract (A) and purified AP-2 protein (B).

DISCUSSION

Previous studies have shown that the level of Na^+/H^+ exchanger mRNA and transcription itself can be increased due to a wide variety of treatments including chronic acid loading (9-11) and treatments causing cell differentiation (8). To date however, there have been few studies on the NHE1 promoter. One of these studies identifies the regions of the human promoter/enhancer region which are capable of binding nuclear factors (6), while the second indirectly examines the role of AP-1 (7). It was suggested that AP-1 could play a role in activating the antiporter especially during acidosis mediated increases in antiporter activity. Alternatively, it was suggested that some other protein kinase C-dependent pathway could mediate the effects of acidosis through AP-1 (7). To date, however, there has been no direct examination of the role of specific transcription factors on the actual NHE1 promoter.

Our analysis suggests that base pairs -95 to -106 play an important role in regulation of expression. Mutation of these base pairs reduced both *in vivo* transcription and binding of nuclear extracts to this region. DNA footprinting also localized to this region. It is likely that the transcription factor AP-2 or a closely related AP-2 like protein is involved in binding to this region. The evidence supporting this is that cotransfection of Hep G2 with an AP-2 expression plasmid increased NHE1 transcription levels. In addition, purified AP-2 protein bound to this sequence and removal of AP-2 protein from nuclear extracts by immunoprecipitation greatly reduced their ability to bind to the AP-2 consensus sequence. However, it is possible that an AP-2 like protein could be responsible for some of the observed effects. Future experiments will attempt to purify the protein binding to this region of the gene. It is of note that base pairs -95 to -105 are perfectly conserved in sequence and location in the human gene (5) while more upstream distal regions are not. This may indicate an important regulatory function. It should be noted that AP-2 is not the only transcription factor involved in regulation of NHE1 expression and other regions have also recently been suggested to be of importance (6).

We examined the mouse NHE1 promoter because of the widespread availability of a number of useful mouse cell lines. Our results show that we have isolated the NHE1 isoform since our probes for screening the genomic library originated from the NHE1 isoform and the clone hybridized under relatively high stringency. We also show that the promoter for the 'housekeeping' isoform of the Na^+/H^+ exchanger family is activated by the transcription factor AP-2 or an AP-2-like transcription factor. Although 'housekeeping' genes such as NHE1 are normally not acutely regulated, there are examples of such promoters that are activated by cis-elements. One such example is the second promoter of the acetyl-CoA carboxylase gene (24) whose activation is mediated through an AP-2-like sequence (25). This example may be analogous to the NHE1 promoter. Both AP-2 and NHE1 transcription rate increase in some models of cellular differentiation. For example, during retinoic acid induced differentiation of human leukemic cells (HL 60), there is an 8.3 fold increase in NHE1 transcription (8). Also, retinoic acid induced differentiation of human NT2 teratocarcinoma cells shows increased AP-2 mRNA and protein expression levels (14). The relationship between increased AP-2 levels during differentiation induced by retinoic acid and the increase in NHE1 transcription seems to be an important issue. Experiments are currently underway which may help to explain this relationship.

The results from this paper have, for the first time, directly examined the role of a specific transcription factor in the regulation of the Na^+/H^+ exchanger gene. We have suggested that this transcription factor is AP-2 or an AP-2-like protein, and we have located the region of the gene where it is able to bind. While the extent to which this protein regulates the Na^+/H^+ exchanger in specific cellular events is not yet known, the identification of a DNA-binding protein motif has provided enough information to warrant further interest. AP-2 is involved in the regulation of a number of cellular events including growth and differentiation (14). The involvement in these events is an interesting characteristic AP-2 has in common with the Na^+/H^+ antiporter (4, 8). Future studies will focus on these specific cellular events and how they work together to control regulation of Na^+/H^+ exchanger levels and growth and differentiation of the cell.

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CHAPTER FOUR

Specific Activation of the Na+/H+ Exchanger Gene During Neuronal Differentiation of Embryonal Carcinoma Cells

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Jason R. B. Dyck and Larry Fliegel.

INTRODUCTION

The Na^+/H^+ exchanger is a mammalian plasma membrane protein that mediates the exchange of intracellular H^+ ions for extracellular Na^+ ions with a stoichiometry of 1:1. Several isoforms of the protein have been identified which are designated NHE1-4. The NHE1 isoform is present in all mammalian cells and has been described as the housekeeping isoform of the Na^+/H^+ exchanger family (1). NHE1 is involved in pH regulation (2), control of cell volume (3), and is activated by growth factors (4). Although the mechanism of regulation of the protein has received much attention, the recent cloning of both the human and the mouse NHE1 genes have enabled more in-depth study of the promoter activity (5, 6; Chapter 3, this document). Specific regions of both promoters have been identified as putative cis-elements that may be responsible for controlling the regulation of the NHE1 gene (6; Chapter 3, this document). We have provided direct evidence of a transcription factor that can upregulate the NHE1 gene. This trans-acting nuclear protein has been identified as the transcription factor AP-2 (5). These findings have shown that the Na^+/H^+ exchanger gene can be upregulated and suggest that the exchanger performs specific functions separate from its normal housekeeping role.

The Na^+/H^+ exchanger is involved in cell proliferation and differentiation (7). During cell proliferation the exchanger is responsible for an elevation of intracellular pH. In some cell types this pH change has been shown to play an important permissive role in growth (8). Cell differentiation, however, results in more drastic alterations in growth patterns and involves the activation of many key genes. How the Na^+/H^+ exchanger gene is regulated and involved during this process remains unknown. Rao *et al.* (7) examined the regulation of Na^+/H^+ exchanger expression and its role in retinoic acid induced differentiation of HL 60 cells. Immediately prior to differentiation into granulocyte-like cells, the activity of the Na^+/H^+ exchanger increases and remains elevated well into differentiation (7, 9). There is an 18-fold increase in NHE1 mRNA levels as well as a 7-fold increase in protein levels (7). This dramatic rise in Na^+/H^+ antiporter levels suggests that Na^+/H^+ exchanger gene expression and differentiation are closely linked.

Retinoic acid induced differentiation is not specific to HL 60 cells. Many murine cell lines can also differentiate when treated with retinoic acid (10). In tissue culture systems the acidic form of vitamin A (retinoic acid) replaces the natural morphogen present during development (11). The effects of retinoic acid are believed to be exerted through a wide variety of receptors and binding proteins. These proteins act as trans-activating factors which can regulate the expression of specific genes (12, 13). The transcription factor AP-2 is one such regulatory protein (14). Retinoic acid causes increases in AP-2 mRNA and protein levels. These increases are believed to be important in initiating differentiation in embryonal carcinoma cell lines such as P19 cells and human NT2 teratocarcinoma cells (14, 15).

P19 cells are a well established tissue culture model for early embryonic determination and differentiation. These cells are a widely utilized pluripotent cell line able to grow continuously in serum-supplemented media. The differentiation of these embryonal carcinoma cells can be controlled by nontoxic doses of retinoic acid or dimethyl sulfoxide. Treatment with retinoic acid effectively induces the development of neurons, astroglia, and microglia cells normally derived from the neuroectoderm. Six days after initial retinoic acid treatment more than 85% of cells express neuronal markers (16). The transcription factor AP-2 has been implicated in retinoic acid induced differentiation of P19 cells and in NT2 cells, a similar human teratocarcinoma cell line (11, 14, 15). In this study we examine regulation of the Na^+/H^+ exchanger gene during retinoic acid induced differentiation of P19 cells. Because AP-2 has been suggested to be important in both regulation of the Na^+/H^+ exchanger gene (6; Chapter 3, this document) and embryonal carcinoma cell differentiation (11, 15) we suspected that AP-2 may be involved in regulation of antiporter expression during differentiation. Our findings provide direct evidence to support the conclusion that the Na^+/H^+ exchanger gene is activated early in P19 cellular differentiation. They suggest that this AP-2 dependent activation may play a key role in the process of retinoic acid induced differentiation in this cell type.

EXPERIMENTAL PROCEDURES

Materials

Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim (Laval, Quebec, Canada) and Bethesda Research Laboratories (Gaithersburg, MD, USA). All trans-retinoic acid and G418 were purchased from Sigma (St. Louis, MO, USA). Hybond-N^+ nylon membranes were obtained from Amersham (Oakville, Ontario, Canada). [α-^{32}P]-dCTP, and [$\gamma^{32}P$]-dATP were from ICN. The plasmid pTZ 19 was from Pharmacia (Piscataway, NJ, USA). All other chemicals were of analytical or molecular biology grade and were purchased from Fisher Scientific (Ottawa, Ontario, Canada), Sigma (St. Louis, MO, USA) or BDH (Toronto, Ontario, Canada).

Cell Culture

P19 mouse embryonal carcinoma cells were obtained from American Type Culture Collection (MD, USA). RAC65 cells were a generous gift from Dr. McBurney from the Department of Medicine, University of Ottawa, Ontario, Canada. Wild type and mutant P19 cells were maintained in alpha minimum essential medium supplemented with 2.5% fetal bovine serum and 7.5% calf serum as reported earlier (17). Before transfection cells were plated in 10 cm dishes at a density of 2.5 X 10^6 cells/ml and incubated for 24 hours. After 24 hours the medium was changed, the cells transfected and incubated for 5 hours. On completion of the incubation stage, the cells were split into two 10 cm dishes; one treated with retinoic acid and the other left in control medium. For the stable cell line, a slightly different protocol was used to induce a high percentage of the cells to differentiate to neuronal cell types. The cells were aggregated for 4 days in the presence of 5 μM retinoic acid in non-adhesive plates then plated on adhesive substrates in the absence of retinoic acid for another 48 hours. NIH 3T3 cells were maintained in DMEM media supplemented with 10 % fetal bovine serum as described earlier (6; Chapter 3, this document).

Reporter Plasmid Construction

pXP-1MP was constructed as described earlier (6; Chapter 3, this document). The 1.1 kb fragment contains base pairs -1085 to +22 of NHE1. To construct pMP+AP2 plasmid, two oligonucleotides were used as primers for polymerase chain reaction (# 1, ttgg atc CGT GAC ACT TCC TTC CCT and #3, cc ttc gaa GGG TCC CGC GGT AGC GGA) were synthesized to amplify base pairs -125 to +22 of the gene. The PCR product had the restriction enzyme sites Bam HI and Hind III generated on either end and was inserted into the Bam HI and Hind III sites of pXP-1. Similarly, pMP-AP2 plasmid was made using the product of the primers # 3 and #2 (#2, ttgg atc CTG CAC CGC GCG GGC GCT) and the 114 bp product (-92 to +22) was inserted into pXP-1. To construct p(AP2)$_3$SV, oligonucleotides AP2R1 (5' GAT C(CT)T TCC TTC CCT GGG CGA CAG GGG CCA 3') and AP2R2 (5' GAT C(CT)G GCC CCT GTC GCC CAG GGA AGG AAA 3') were annealed, ligated together and then digested with restriction enzymes Bam HI and Bgl II. The product was size fractionated on a 9 % acrylamide gel, and the ligated product of the oligonucleotides was purified and ligated into the Bam HI site of PTZ 19. The DNA was removed from PTZ with the restriction enzymes Sal I and Eco RI. The SV40 promoter was digested from pCAT-promoter plasmid (Promega) using Eco RI and Hind III and was ligated into the corresponding sites of pBluescript SK- (Stratagene, LaJolla, CA, USA). This fragment was removed from pBluescript SK- with the restriction enzymes Eco RI and Hind III. The plasmid pXP-1 was digested with Hind III and Sal I and ligated simultaneously with the two fragments: the Sal I and Eco RI fragment containing the AP-2 repeats and the Eco RI and Hind III fragment containing the SV40 promoter. The resulting plasmid (p(AP2)$_3$SV) contained three tandem copies of the AP-2 site of the mouse NHE1 promoter located 5' to the SV40 promoter. All plasmids were sequenced to verify proper orientation and fidelity of PCR.

Transfection and Reporter Assays

Cells were transiently transfected using the calcium phosphate precipitation method as described earlier (6; Chapter 3, this document). P19 cells were plated onto 10 cm dishes and each dish received 10 µg of luciferase reporter plasmid and 10 µg of pSV-ß-Galactosidase plasmid as an internal control. After cells were incubated for 5 hours with the plasmid and the calcium phosphate co-precipitate, they were divided into new plates with a dilution of 1:2 and were treated with 5 µM of retinoic acid or control medium. After 40 hours cells were harvested and cell lysates assayed for luciferase activity and ß-Galactosidase activity. NIH 3T3 cells were transfected as described earlier (6; Chapter 3, this document). Stably transfected cells were made using the same calcium phosphate transfection protocol with pRSVneo plasmid (1 µg) and with pMP+AP2 (20 µg). After transfection, cells were incubated for 24 hours before being replated at a dilution of 1:10. After replating the cells were treated with 200 µg/ml of G418 for 18 days. G418 resistant colonies were identified and individual colonies were repropagated until reaching a higher density. They were then assayed for the ability to express luciferase activity. Polymerase Chain Reaction was used to determine if the entire mouse promoter region from pMP+AP2 had been inserted. The PCR primers amplified a region from the 5' end of the luciferase gene to the 5' end of the mouse NHE1 insert. P19 cells stably transfected with the plasmid pMP+AP2 (referred to as P19A2) were used to study cell differentiation. P19A2 cells were aggregated for 4 days on 10 cm non-adhesive type petri dishes in the presence of retinoic acid then plated on adhesive substrates for another 48 hours. The same number of cells were harvested at various time points throughout the differentiation process and were assayed for luciferase activity. Each luciferase assay contained 40 µl of the cell lysate and 100 µl of the luciferase assay reagent (Tricine 20 mM, Magnesium Carbonate 1.07 mM, $MgSO_4$ 2.67 mM, EDTA 0.1 mM, DTT 33.3 mM, luciferin 470 mM, ATP 530 mM, Coenzyme A 270 mM, and BSA 1 ng/ml). The ß-Galactosidase assay included 50 µl of cell lysate, 50 µl H_2O and 20 µl of O-Nitrophenyl-ß-D-galactopyranoside, incubated at 37°C for 60 minutes. Luciferase activity was assayed with an LKB luminometer and normalized to ß-Galactosidase for efficiency of transfection for transiently transfected cells and to the number of cells and protein content for stably transfected cells. Results are reported as mean ± SE; where not shown the SE was too small to be displayed.

Polyacrylamide Gels and Immunostaining

SDS/PAGE was on 9% polyacrylamide gels as described earlier (18). Protein levels from cell extracts were determined using the Bio-Rad DC Protein Assay kit with BSA as the standard. Equal amounts of protein were loaded onto the gels and after separation, proteins were transferred electrophoretically on to nitrocellulose membranes. Immunostaining of nitrocellulose was carried out in the presence of 1% skim milk powder using the neuron specific class III ß-tubulin monoclonal antibody (19). The antibody was detected using the Amersham Enhanced Chemiluminescence Western Blotting and Detection System as described by the manufacturer.

Internal pH measurement

For measurement of intracellular pH P19 cells were grown for 40 hours with or without retinoic acid and then trypsinized and resuspended in PBS. Fluorescence measurements were made on a Shimadzu RF-5000 spectrofluorophotometer. Cells were incubated in a cuvette with constant stirring. The acetoxy methyl ester of 2'-7'-bis (2-carboxyethyl)- 5(6)-carboxyfluorescein (BCECF-AM 2 µg/ml) was incubated with cells for 5 minutes in a solution containing 135 µM NaCl, 5 µM KCl, 1.8 µM $CaCl_2$, 1.0 µM $MgSO_4$, 5.5 µM Glucose, 10 µM HEPES, pH 7.4. Intracellular pH was measured utilizing the dual excitation single emission ratio technique. Excitation wavelengths were at 452 and 500 nM with emission at 520 nM. This allows measurement of intracellular pH that is independent of cell concentration and dye loading (20). A calibration curve for intracellular dye was generated by the nigericin method (20). To measure initial rates of proton extrusion after an acid challenge the NH_4Cl prepulse method was used. Cells were incubated with buffer containing 15 μM NH_4Cl for 5 min. They were pelleted (10,000 rpm for 30 seconds) and then resuspended in Na^+ free buffer that contained 135 mM N-methyl-D-glucamine instead of NaCl. The cells pelleted and resuspended in a solution containing 67.5 mM NaCl (and 67.5 mM N-methyl-D-glucamine) and the initial rate of rise of internal pH was measured during the first 40 seconds of recovery.

DNA Binding Assays

Nuclear extracts were prepared from P19 cells as reported by Schreiber *et al.* (21). The synthetic oligonucleotides of the sequence 5' TTC CTT CCC TGG GCG ACA GGG GCC 3' (MPAP2a) and 5' GGC CCC TGT CGC CCA GGG AAG GAA 3' (MPAP2b) were made which correspond to the AP-2 region of the mouse Na^+/H^+ exchanger promoter. The oligonucleotides were end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. They were heated to 95°C for 2 minutes and cooled to room temperature overnight for annealing. DNA binding reactions were for 10 minutes at room temperature and contained: 30 000 CPM of [γ-^{32}P]-labeled oligonucleotides mixed with P19 nuclear extract (5 μg) in a binding buffer (4% glycerol, 1.25 μM $MgCl_2$, 0.5 μM EDTA, 0.5 μM DTT, 50 μM NaCl, 10 μM Tris-HCl, pH 7.5 and 0.05 μg/ml poly dI:dC). After electrophoresis on 4% polyacrylamide gels the gels were dried and exposed to X-ray film for 16 hours. In some experiments nuclear extracts were treated to specifically remove AP-2 protein before gel mobility shift assays were performed. Twenty µl of a 10% solution of Protein-A was incubated with 1 or 2 µg of AP-2 antibody (IgG, Santa Cruz Biotechnology Inc.) for 1 hr at 4°C. After 1 hour the solution was washed 3 times (20 µM Tris, pH 7.9, 400 µM NaCl, 1 µM EDTA, 1 µM EGTA, 1 µM DTT, and 1 µM PMSF), mixed with 5 µl of P19 nuclear extracts and incubated at 4°C for 16 hours. The mixture was then spun at 6,500 rpm for 10 min. and the supernatants were collected. Control experiments contained equal amounts of irrelevant IgG.

RESULTS

Identification of Regulatory Regions in the Mouse NHE1 Gene.- A 1.1 kb fragment of the mouse NHE1 gene has recently been cloned and putative regulatory sites have been identified (6; Chapter 3, this document). Initial studies concentrated on identifying the regions of the promoter that are involved in the regulation of the Na^+/H^+ exchanger gene in P19 cells. We constructed several different plasmids containing varying fragments of the promoter/enhancer region. Our initial construct contained the entire 1.1 kb mouse promoter/enhancer region located 5' to a luciferase gene. This plasmid was termed pXP-1MP. From this initial plasmid we deleted base pairs -1085 to -126 (pMP+AP2) and -1085 to -92 (pMP-AP2) to create two new plasmids (Figure 4-1). These plasmids were then transfected into P19 cells in an attempt to determine which regions were responsible for regulating the NHE1 gene. All transfection experiments also included pSV-ß-Galactosidase which was used to normalize for any differences in transfection efficiency. Figure 4-1 is a comparison of luciferase activity between the three plasmids. When the region from -1085 to -126 was deleted from the original pXP-1MP construct there was a 37% decrease in the rate of transcription. This decrease indicates that the deleted region must contain some element(s) that contribute to the basal levels of transcription of the NHE1 gene in P19 cells. We then examined the importance of the region immediately 3' to this deletion. When an extra 33 base pairs were deleted from pMP+AP2 the level of luciferase activity decreased another 62% from that seen with the complete promoter. These 33 base pairs have been shown earlier to contain the binding site for the transcription factor AP-2 and to be involved in NHE1 gene regulation (6; Chapter 3, this document).

To examine the direct involvement of the AP-2 site from the mouse NHE1 gene in P19 cells, two new reporter plasmids were constructed. The luciferase gene was linked to a wild type SV40 promoter (pSVLUC), or to the SV40 promoter which contained a triplicated AP-2 binding site derived from the NHE1 gene ($p(AP2)_3SV$; Figure 4-2). These plasmids were then transfected into P19 cells with pSV-ß-Galactosidase. Figure 4-2A demonstrates the importance of the AP-2 site in the regulation of transcription in P19 cells. In these experiments, $p(AP2)_3SV$ showed a 3.2-fold increase in transcription in comparison to pSVLUC (Figure 4-2A, lanes 1 and 2). This indicates that in P19 cells the AP-2 site from the NHE1 gene can activate a foreign promoter and increase the luciferase levels. Because the two new plasmids differ only in the presence of the NHE1 AP-2 site, we can conclude that this site plays a significant role in the elevated expression of the exchanger gene in P19 cells. This regulation may allow for a constitutively higher level of transcription in P19 cells compared to cells with lower levels of AP-2 protein. The need for increased exchanger promoter activity may be due to the pluripotent nature of

P19 cells (17). Higher levels of NHE1 transcription may lead to a parallel increase in protein expression. This overall increase in exchanger protein may be important for the P19 cell to maintain its elevated pH (22). The increase in steady state pH in P19 cells may contribute to producing a permissive environment for protein and DNA synthesis necessary for differentiation (23, 24).

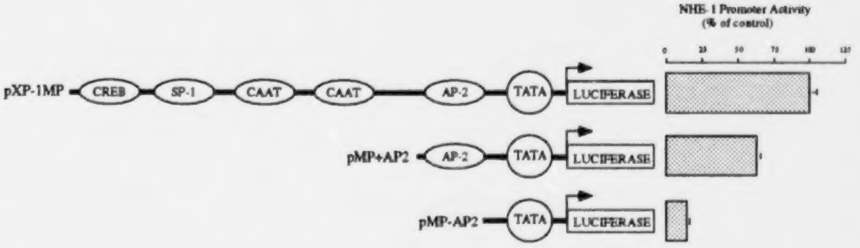
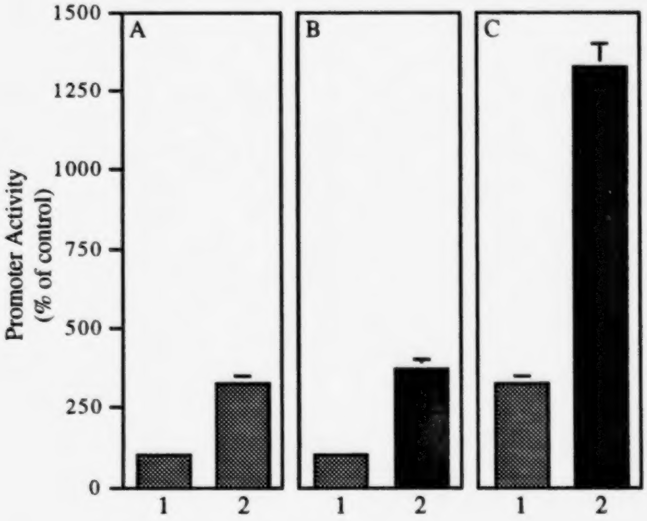
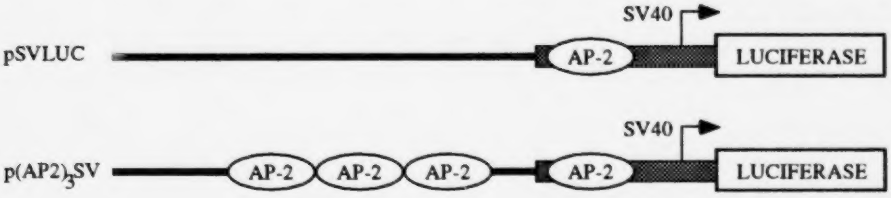


Figure 4-1. **NHE1 promoter activity in mouse P19 embryonal carcinoma cells.** Mouse P19 cells were transiently transfected with either pXP-1MP, pMP+AP2 or pMP-AP2. pXP-1MP contains base pairs -1085 to +22 of the mouse promoter/enhancer region. pMP+AP2 contains the region between -125 and +22 and pMP-AP2 contains the region between -92 and +22. Ovals indicate putative binding sites for DNA-binding proteins, SP-1, CAAT, CREB and AP-2 and the circle represents the TATA box. Luciferase activity was corrected for transfection efficiency using cotransfection with pSV-ß-Galactosidase. For all transfection experiments the results are mean ± SE from at least three independent experiments each carried out in triplicate utilizing at least two different DNA preparations for each plasmid.

Figure 4-2. **AP-2 dependent activation of the SV40 promoter.** Plasmids pSVLUC and p(AP2)$_3$SV were transiently transfected into P19 cells and either treated with retinoic acid to induce differentiation or untreated for controls. Corrected levels of luciferase activity are shown. (A), promoter activity from pSVLUC (lane 1) and p(AP2)$_3$SV (lane 2) in P19 cells. (B), promoter activity from pSVLUC in P19 cells (lane 1) and in P19 cells that had been treated with 5 μM retinoic acid for 40 hours (lane 2). (C), promoter activity from p(AP2)$_3$SV in P19 cells (lane 1) and in P19 cells that had been treated with 5 μM retinoic acid for 40 hours (lane 2). pSV-ß-Galactosidase was used as an internal control for all transfections. For all experiments the results reported were obtained from at least three independent experiments each carried out in triplicate using at least two different DNA preparations for each plasmid. Also, a schematic representation of the luciferase reporter plasmids used in the experiments is shown. The dotted box indicates the SV40 promoter, where the arrows denote the direction and start sites of transcription. The single oval indicates the SV40 AP-2 consensus sites and the three linked ovals represent three copies of the NHE1 AP-2 consensus site.


1500
1250
1000
750
500
250
0
Promoter Activity
(% of control)
A
B
C
1
2
1
2
1
2

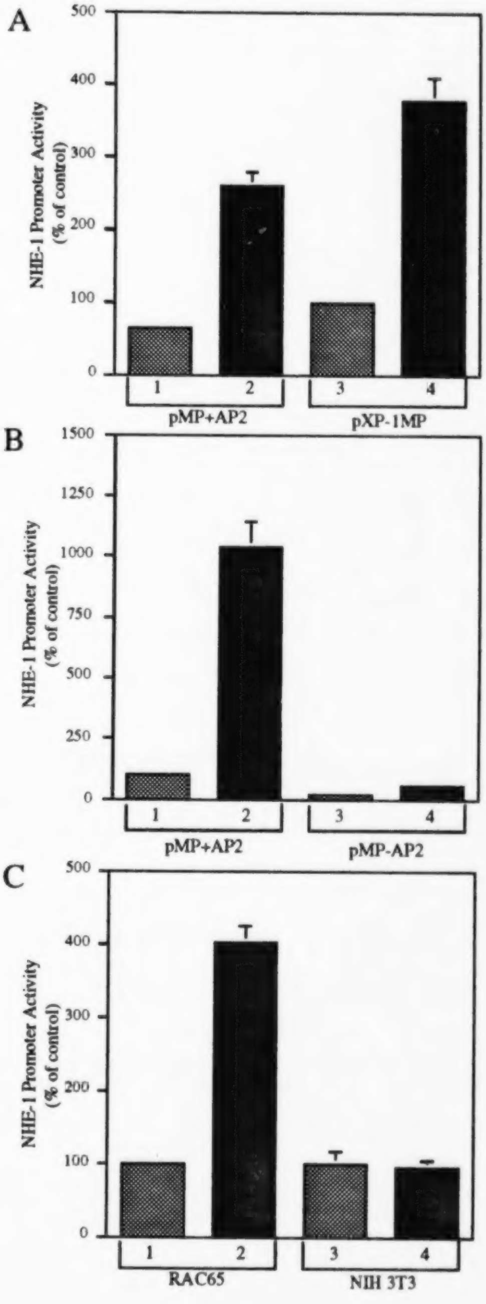

SV40
pSVLUC
AP-2
LUCIFERASE
SV40
p(AP2)3SV
AP-2
AP-2
AP-2
AP-2
LUCIFERASE

Regulation of The Gene During Retinoic Acid Induced Differentiation- Our hypothesis is that the AP-2 site is involved in regulating the mouse NHE1 gene in differentiating P19 cells. To test this hypothesis we first needed to rule out any involvement from the region(s) proximal to the AP-2 site. Because this proximal region is involved in regulating basal levels of transcription in P19 cells (Figure 4-1), the same region may also be involved in regulating the gene during differentiation. P19 cells were transfected with either pXP-1MP or with pMP+AP2. The cells were then treated with 0.5 μM all-trans retinoic acid for 40 hours. This concentration of retinoic acid has been shown to induce differentiation of P19 cells into neurons, astrocytes and fibroblast-like cells (25). Figure 4-3A demonstrates the effects of differentiation on the level of transcription of the NHE1 promoter. After 40 hours of treatment the differentiating cells exhibited a 4-fold increase in luciferase activity (Figure 4-3A). The results show that retinoic acid-induced differentiation results in an increase in transcriptional activity from the mouse NHE1 gene. Also, because the amount of increase in luciferase activity is the same in both plasmids (i.e., pXP-1MP and pMP+AP2) then the region responsible for the increase must not lie within base pairs -1085 to -126. It is for this reason we concentrated further studies on the region of the gene beginning from the AP-2 site to the start site of transcription.

When P19 cells are induced to differentiate with retinoic acid, cells are subjected to at least two variables. The increase in NHE1 transcription may be directly due to retinoic acid, due to differentiation, or to a combination of the two stimuli. To ensure that a maximal stimulus has been achieved and complete neuronal differentiation has been induced, we increased the concentration of retinoic acid to 5 μM. This concentration has been shown to be sufficient to fully induce neuronal differentiation (26). With higher concentrations of retinoic acid we observed even higher levels of NHE1 promoter activity. Figure 4-3B demonstrates that the construct pMP+AP2 now produces a 10-fold increase in luciferase activity with retinoic acid induced stimulation. The plasmid pMP-AP2, however, produced only a minor, insignificant increase in promoter activity compared with pMP+AP2. The results from Figure 4-3B confirm that retinoic acid induced effects on P19 cells are localized to the region known to interact with transcription factor AP-2 (6; Chapter 3, this document). Also, Figure 4-3B demonstrates that with the higher concentration of retinoic acid either the cells are stimulated to further differentiate or that retinoic acid itself affects the NHE1 gene.

In an attempt to distinguish direct transcriptional effects of retinoic acid from the effects mediated through differentiation, the P19 mutant RAC65 was utilized. This cell line was derived from a clone which has been selected for its inability to differentiate when exposed to retinoic acid (27). These cells were transfected with pMP+AP2 and pSV-ß-Galactosidase and then treated either with or without 5 μM retinoic acid. When cells were treated with retinoic acid there was a 4-fold increase in transcription from the NHE1 promoter (Figure 4-3C, lanes 1 and 2). From this, one can conclude that of the 10-fold increase in transcription seen in Figure 4-3B, approximately 40 % is due to direct or related effects of retinoic acid and 60 % is due to effects mediated via differentiation. To confirm that the effect of retinoic acid on the promoter in P19 cells is specific, NIH 3T3 cells were transfected with pMP+AP2 and treated with 5 μM retinoic acid (Figure 4-3C). Since NIH 3T3 cells do not differentiate, any effect seen with retinoic acid would indicate a direct response of the promoter to retinoic acid.

Figure 4-3. **Effects of retinoic acid induced differentiation on transcription by the NHE1 promoter in P19 and RAC65 cells.** (A), luciferase activity from pMP+AP2 and pXP-1MP in control P19 cells (lanes 1 and 3) and cells induced to differentiated with 0.5 μM retinoic acid (lanes 2 and 4). (B), luciferase activity from pMP+AP2 and pMP-AP2 in P19 cells (lanes 1 and 3) and cells induced to differentiate with 5 μM retinoic acid (lanes 2 and 4). (C), luciferase activity from pMP+AP2 in untreated RAC65 and NIH 3T3 cells (lanes 1 and 3) and in RAC65 and NIH 3T3 cells treated with 5 μM retinoic acid (lanes 2 and 4). pSV-ß-Galactosidase was used as an internal control. Results are from at least three independent experiments carried out in triplicate using at least two different DNA preparations for each plasmid.


A
NHE-1 Promoter Activity (% of control)
0
100
200
300
400
500
1
2
3
4
pMP+AP2
pXP-1MP
B
NHE-1 Promoter Activity (% of control)
0
250
500
750
1000
1250
1500
1
2
3
4
pMP+AP2
pMP-AP2
C
NHE-1 Promoter Activity (% of control)
0
100
200
300
400
500
1
2
3
4
RAC65
NIH 3T3

As seen in Figure 4-3C (lanes 3 and 4), there is no change in NHE1 promoter activity. This indicates that the NHE1 gene is not directly responsive to retinoic acid in all cell types, but may be specific to differentiating cells.

Activation of the NHE1 Gene Occurs at an Early Stage of P19 Differentiation- When P19 cells are treated with retinoic acid, a large number of events occur which cause differentiation. Many regulatory genes are expressed in a temporal manner. However, it is not known if a cascade of gene expression occurs (13). How the Na^+/H^+ exchanger fits into the process of differentiation is also not known. Our next experiment set out to determine at what point in differentiation the transcriptional activity of the NHE1 gene is increased. We subjected P19 cells to 5 μM retinoic acid for 40 hours as described above. We then analyzed the cellular extracts using Western immunoblot analysis (Figure 4-4). An antibody produced against ß-tubulin was used as an indicator of differentiation into neuronal cells. As seen in Figure 4-4A, control cells and cells treated with retinoic acid for 40 hours express the same low levels of ß-tubulin in the early stages of differentiation. This low level of ß-tubulin is produced from the small fraction of P19 cells known to differentiate spontaneously when plated at high concentrations (17). Because retinoic acid treated cells express the same level of ß-tubulin as the controls, it appears that 40 hours of retinoic acid exposure is not sufficient to produce fully differentiated cells. This does not mean, however, that the retinoic acid treated cells have not begun the differentiation process. The effects of retinoic acid have been shown to produce cellular alterations after as little as 2-4 hours of exposure (16). In addition we observed dramatic stimulation of the NHE1 gene within this time (Figure 4-3).

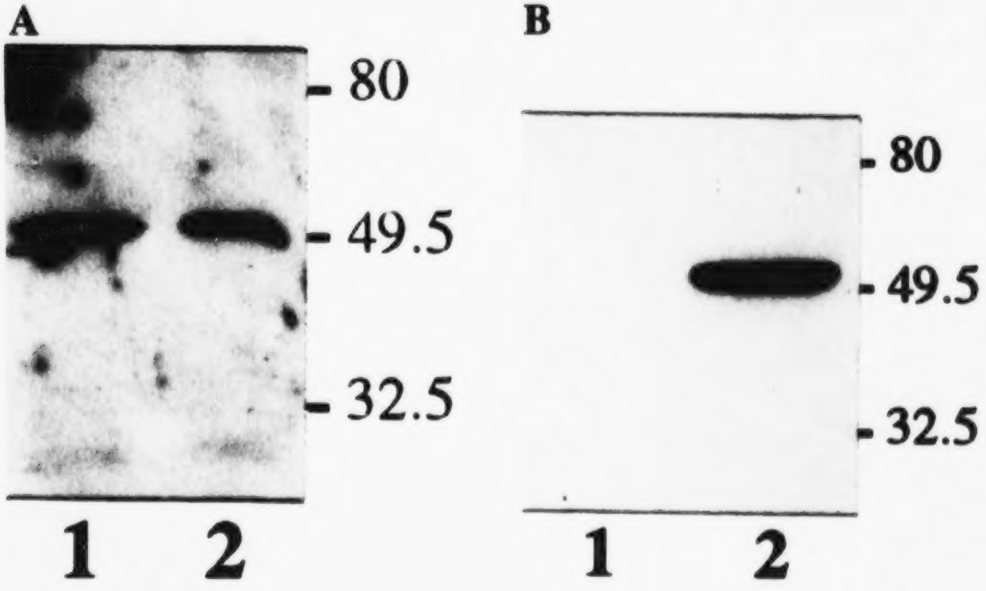


Figure 4-4. **Western immunoblot analysis of P19 cells with ß-tubulin antibody.** Cell extracts from control and retinoic acid treated P19 cells (5 μM) were run on a 9% SDS PAGE gel. After transfer to Nitrocellulose samples were immunostained with class III ß-tubulin antibody. (A), lane 1 indicates the untreated P19 cells and lane 2 represents cells treated with retinoic acid for 40 hours. The immunoblot was developed using the Amersham enhanced chemiluminescence kit and was exposed for 2 minutes to enhance detection of the weak signal. (B), Control and retinoic acid treated P19 cells immunostained with ß-tubulin antibody, lane 1 indicates control P19 cells and lane 2 represents cell extracts treated with retinoic acid for 4 days using the differentiation protocol to enhance development of neuronal cells as described in "Experimental Procedures". The immunoblot was exposed for 10 seconds.

It can therefore be assumed that differentiation has been initiated in retinoic acid treated cells and it is at this point where the Na^+/H^+ exchanger gene is upregulated. To confirm that our treatment of P19 cells does result in differentiation we analyzed cells that had undergone the enhanced neuronal differentiation protocol as described in "Experimental Procedures". The results are shown in Figure 4-4B. Longer treatment of aggregated P19 cells with retinoic acid resulted in a strong increase in the levels of expression of ß-tubulin in comparison to controls.

Because transient transfection of P19 cells only allowed for the study of events occurring early in the differentiation process, a separate procedure was used to examine regulation of the NHE1 gene during the later stages of neuronal differentiation. P19 cells were stably transfected with the plasmid pMP+AP2. This stable cell line (P19A2) was treated to induce a high percentage of the cells to differentiate to neuronal cell types. The cells were aggregated for 4 days in the presence of retinoic acid in non-adhesive plates then plated on adhesive substrates for another 48 hours. This treatment produces cells that begin to lose embryonic carcinoma antigen properties shortly after 48 hours (28). Also, by day six, approximately 85% of the cells begin to express specific neuronal markers (16; Figure 4-4B). Equal numbers of cells were harvested at various time points during differentiation and assayed for luciferase activity. Figure 4-5 illustrates the activity of the NHE1 promoter during this differentiation procedure. Within 48 hours the promoter activity increased 19.4-fold, thus confirming similar results seen with the transient transfection protocol.

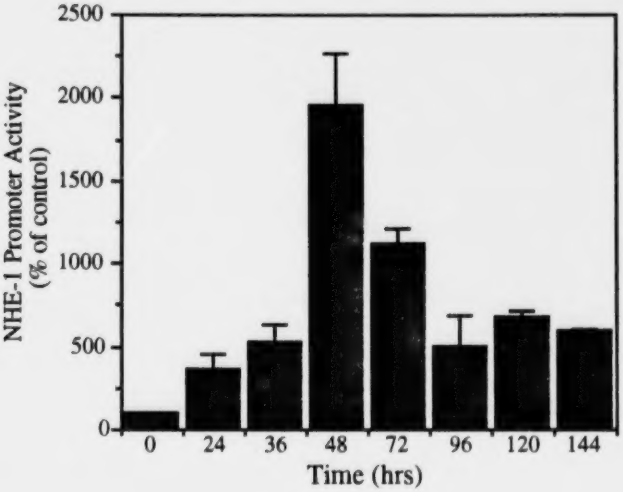
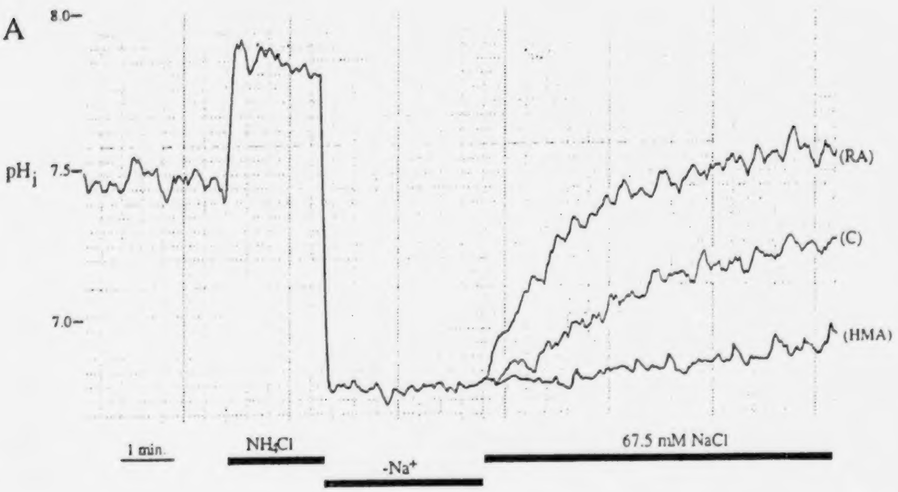


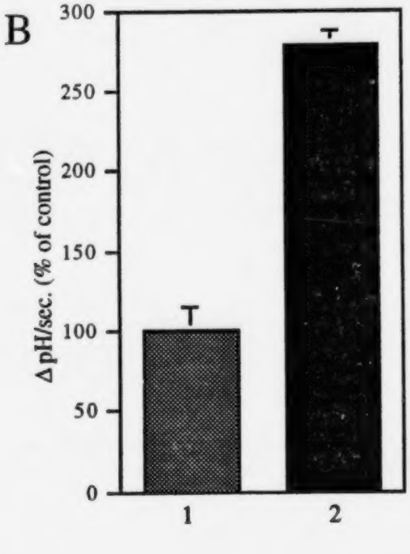
Figure 4-5. **NHE1 promoter activity during neuronal differentiation of P19 cells.** P19 cells were stably transfected with the NHE1 promoter plasmid, pMP+AP2 and the neomycin gene (pSVneo). The stable transformant (P19A2) cells were then incubated with 5 μM retinoic acid for 4 days and treated to enhance neuronal differentiation as described in "Experimental Procedures". After 4 days of retinoic acid treatment the cells were then plated and incubated for 2 days further in the absence of retinoic acid. Equal numbers of cells were harvested at time 0, and after 24, 36, 48, 72, 96 120, and 144 hours.

During days 3 and 4 a dramatic decrease in luciferase activity was observed. Since it is not until after this time that most of the cells have begun expressing neuronal markers, it can be concluded that the initial increases in NHE1 promoter activity are not due to changes in cell type. The alterations in promoter activity must therefore be a result of early events involved in differentiation. This result suggests that there may be a need to alter intracellular pH or change the capacity to regulate intracellular pH at a very early stage in differentiation.

To assess if the activity of the Na^+/H^+ exchanger protein increases during differentiation we measured the activity of the exchanger in both control P19 cells and P19 cells treated with retinoic acid to induce differentiation (Figure 4-6). The control and the retinoic acid treated cells were subjected to an acute acid load induced by ammonium chloride. Figure 4-6A shows a tracing of a typical experiment for P19 cells. The rate of recovery from ammonium chloride induced acid load of the retinoic acid treated cells is superimposed. It is apparent that the retinoic acid induced differentiating cells recovered at a much greater rate than the P19 control group. The summary of the differences in the initial rate of recovery for an acid load is shown in Figure 4-6B. The retinoic acid treated cells recovered at a rate approximately 3 times greater than untreated cells. The recovery from acid load was inhibited by the amiloride analog hexamethylamiloride, indicating Na^+/H^+ exchanger mediated pH changes. This suggests that there is a significant increase in Na^+/H^+ exchanger activity in differentiating P19 cells. Because the Na^+/H^+ exchanger in P19 cells has previously been shown to be constitutively active and unaffected by extracellular stimuli (22), the most plausible

Figure 4-6. **Recovery from an acute acid load by control and retinoic acid treated P19 cells.** (A), cells were prepared and intracellular pH was measured as described in "Experimental Procedures". Ammonium chloride prepulse was used to induce acute acidosis. The intracellular pH and recovery of control (C) cells from an acute acid load in 67.5 mM NaCl are shown. The recovery is also shown from the retinoic acid (RA) treated cells. Inhibition of recovery by hexamethylamiloride is shown (HMA). (B), P19 cells (lane 1) and retinoic acid treated cells (lane 2) were examined as described in (A). The initial rate of recovery in the presence of 67.5 mM NaCl and 67.5 mM N-methyl-D-glucamine is shown. Each group represents the means (± SE) of at least 6 independent experiments.


A
8.0
pH$_i$
7.5
7.0
(RA)
(C)
(HMA)
1 min.
NH$_4$Cl
-Na$^+$
67.5 mM NaCl


B
300
250
200
150
100
50
0
Δ pH/sec. (% of control)
1
2

explanation is that increased promoter activity results in elevated exchanger levels.

The Role of Transcription Factor AP-2 in the Regulation of the NHE1 Gene During Retinoic Acid Induced Neuronal Differentiation- The activity of the NHE1 promoter has been shown to increase upon stimulation of P19 cells with retinoic acid. The large increase in transcription can be attributed in part to a 33 base pair region located at -125 to -93 of the mouse NHE1 gene. This region has previously been shown to bind AP-2 protein and regulate the Na^+/H^+ exchanger gene (6). Because the levels of AP-2 protein have been shown to increase in P19 cells with retinoic acid induced differentiation (15), we investigated the role of AP-2 in regulating NHE1 during this time. The constructs pSVLUC and $p(AP2)_3SV$ were used to demonstrate the importance of AP-2 in regulating differentiation. Examination of the sequence of the SV40 promoter shows the presence of one AP-2 like site. The plasmid containing the SV40 promoter (pSVLUC) showed a 3.7-fold increase in transcription when the cells are induced to differentiate (Figure 4-2B). This result indicates that an independent viral promoter that contains its own AP-2 site is also responsive to retinoic acid induced differentiation. This finding is of particular importance because it indicates that the AP-2 dependent transcriptional stimulation observed during retinoic acid induced differentiation may not occur exclusively in the NHE1 gene but may occur in other genes. We showed that the insertion of 3 tandem repeats of the NHE1 AP-2 site into pSVLUC ($p(AP2)_3SV$) results in an increase in basal levels of transcription in P19 cells (Figure 4-2A). This increase may be due to the increased copies of the AP-2 containing region responding to basal levels of AP-2 protein in the cell. When $p(AP2)_3SV$ is transfected into P19 cells and the cells are induced to differentiate with retinoic acid, we demonstrate a large increase in promoter activity. The increase is approximately 4-fold greater than untreated p(AP2)$_3$SV and over 13-fold greater than the untreated levels of transcription from the plasmid pSVLUC (Figure 4-2B and 4-2C). The absolute level of stimulation of luciferase activity from p(AP2)$_3$SV in the presence of retinoic acid is also much greater than that of pSVLUC stimulated with retinoic acid (Figure 4-2B, 4-2C). It appears that the repeated AP-2 site from the NHE1 gene can further stimulate transcriptional activity in P19 cells in response to retinoic acid induced differentiation. This shows that the increased number of AP-2 sites can respond to retinoic acid stimulation with greater effectiveness in comparison to the SV40 promoter alone.

Bandshift experiments were used to confirm the identity of the transcription factor regulating the NHE1 gene during retinoic acid induced differentiation. We used a 24 base pair double stranded oligonucleotide (MPAP2a,b) which was previously shown to bind specifically to purified AP-2 protein and AP-2 protein from NIH 3T3 nuclear extracts (6; Chapter 3, this document). Nuclear extracts from both control and retinoic acid treated P19 cells were isolated and incubated with the oligonucleotide MPAP2a,b. AP-2 protein from nuclear extracts of treated and untreated P19 cells caused a shift to occur. Nuclear extracts from the retinoic acid-induced cells, however, produced a considerably larger increase in the level of shifted oligonucleotide (Figure 4-7A). This indicates an increase in the amount of AP-2 protein binding to the oligonucleotide compared to the amount seen with the control P19 cells. This agrees with the recent study indicating increased AP-2 protein levels in retinoic acid induced differentiating P19 cells (15).

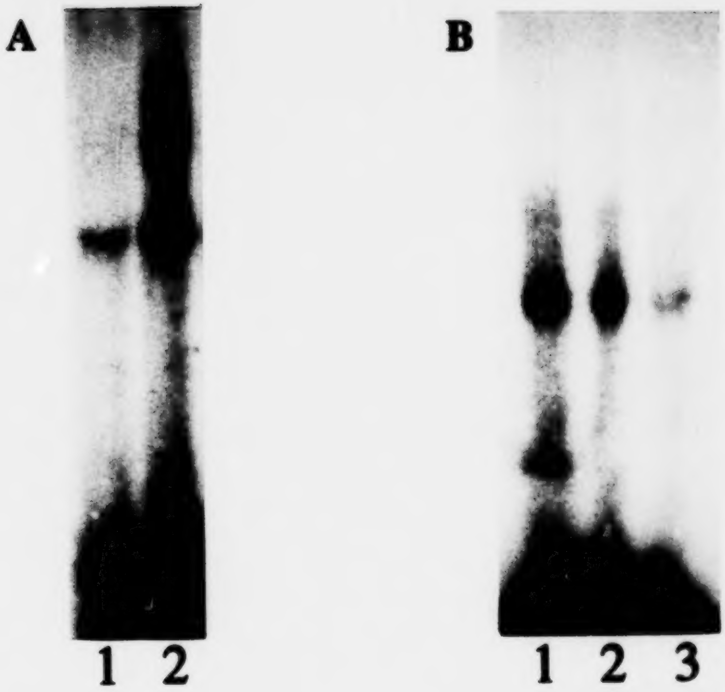


Figure 4-7. **DNA-mobility shift binding assay of the mouse NHE1 AP-2 site in control and retinoic acid differentiated P19 cells.** The labeled 24 base pair oligonucleotide MPAP2a,b (positions -117 to -94) was incubated for 10 minutes at room temperature with nuclear extracts from control or retinoic acid treated P19 cells. The binding mixtures were analyzed by electrophoresis on 4% polyacrylamide gels as described in the "Experimental Procedures". (A), Lane 1 contains 5 μg of nuclear extract from control P19 cells, lane 2 contains 5 μg of nuclear extract from retinoic acid treated P19 cells. (B), DNA-mobility shift binding assay was performed with nuclear extracts from retinoic acid treated P19 cells that were extracted via immunoprecipitation with either 2 μg of irrelevant IgG (lane 1), 1 μg of anti-AP-2 antibody (lane 2) or 2 μg of anti-AP-2 antibody (lane 3).

To confirm that the shifted band was due to AP-2 protein, AP-2 was specifically removed from the nuclear extracts by immunoprecipitation with anti-AP-2 antibody (Figure 4-7B). Nuclear extracts from retinoic acid treated P19 cells were immunoprecipitated with either 2 μg of irrelevant IgG (lane 1), 1 μg of anti-AP-2 containing IgG (lane 2) or 2 μg of anti-AP-2 containing IgG (lane 3). Control IgG had no effect on the binding of AP-2 to the oligonucleotide whereas anti-AP-2 antibody greatly reduced binding to the oligonucleotide in a dose dependent manner. This indicates that AP-2 protein from P19 cells binds to the oligonucleotide MPAP2a,b, and produces the resulting shift. These results provide strong evidence to support the conclusion that transcription factor AP-2 is increased in differentiation of P19 cells and that this increase acts to regulate the NHE1 gene during cellular differentiation.

DISCUSSION

Differentiation of mammalian cells is a complicated process involving many steps and the complex regulation of gene expression. It has been possible to study elements of neuronal differentiation with the advent of specific teratocarcinoma cell lines such as P19. In these embryonal carcinoma cells the expression of several key regulatory genes occurs in an appropriate defined sequence during retinoic acid induced differentiation (13). Regulatory proteins involved in the differentiation into neurons include the retinoic acid receptors, epidermal growth factor receptor and the transcription factors Oct-3 and Brn-2 (13). Recently, the transcription factor AP-2 has been shown to increase during differentiation of P19 cells and during differentiation of a similar human teratocarcinoma cell line (11, 14, 15). This suggests that AP-2 may play an important role in regulation of neuronal differentiation. We have earlier shown that AP-2 is important in regulation of NHE1 transcription (6; Chapter 3, this document). It has also been demonstrated that there is a dramatic increase in NHE1 message levels in retinoic acid induced differentiation of HL 60 cells (7). Therefore we suspected a close relationship between retinoic acid induced cellular differentiation, the Na^+/H^+ exchanger and the transcription factor AP-2.

The results of our present study confirm that differentiation of P19 cells is linked with Na^+/H^+ exchanger expression. Transcription of the Na^+/H^+ exchanger is dramatically increased during neuronal differentiation of P19 cells (preliminary results indicated that NHE1 mRNA levels also increase). When RAC65 cells were stimulated with retinoic acid the response of the NHE1 promoter was attenuated by 60 % in comparison to P19 cells. This cell line is believed to possess a dominant repressor mutation of the retinoic acid receptor a gene (27). The mutation blocks the process of differentiation and may block the actions of all three classes of retinoic acid receptor α, ß, and γ (13). Because the NHE1 gene was only partially activated in RAC65 cells this suggests that the increase comes about as a result of two components of retinoic acid stimulation. One of these stimuli is retinoic acid and the other is the cellular process of differentiation. Differentiation may act to activate NHE1 expression through other trans-acting elements downstream of retinoic acid. Clearly at least one of these downstream elements activated by retinoic acid is the transcription factor AP-2. The response of the NHE1 gene to retinoic acid was eliminated when the AP-2 containing region was deleted. Also, insertion of the AP-2 containing region into a foreign promoter resulted in a similar response to retinoic acid stimulation. With the stimulation of P19 cells by retinoic acid, we have also shown an increase in the amount of AP-2 protein binding to this region. This may be due to elevated AP-2 levels arising from increased transcription, similar to what has been reported in P19 cells and in other teratocarcinoma cell lines with retinoic acid treatment (14, 15). An alternative, yet less likely scenario, suggests that the increase in AP-2 binding may be due to increased phosphorylation of the transcription factor thus allowing for increased DNA binding properties. In either case, the net effect is a stimulation of transcription from the NHE1 promoter. It may be that short term initial effects of retinoic acid are mediated through phosphorylation of AP-2 and that other elements of stimulation require elevation of AP-2 levels. Retinoic acid has been shown to stimulate rapid phosphorylation of several proteins upon administration to HL 60 cells (29, 30). In addition it has been suggested that the transcription factor AP-2 may be regulated by phosphorylation in some cell types (31). Future experiments will investigate these possibilities.

In intact RAC65 cells the response to retinoic acid was attenuated. However, the cells still responded with a 4-fold increase in NHE1 transcription. Because the response of the Na^+/H^+ exchanger gene was only partially attenuated there must be some other mechanism by which retinoic acid stimulation activates this gene without the process of differentiation and the resulting cascade of cellular events. At the present time the mechanism by which this occurs is not known. It is possible that retinoic acid acts acutely to regulate the NHE1 gene by a mechanism such as phosphorylation of AP-2 and that differentiation is required for further activation. It has yet to be determined whether this is what occurs in retinoic acid stimulation of RAC65 cells.

We also found that activation of the NHE1 promoter is an early specific event in retinoic acid stimulation of neuronal differentiation of P19 cells. Retinoic acid treatment of P19 cells or RAC65 cells resulted in large increases in NHE1 activity after only 40 hours but had no effect on NIH 3T3 cells. The effects on P19 cells occurred before marked changes in neuronal markers such as ß-tubulin. The results obtained from the stable cell line, P19A2, confirm this observation. The peak activation of the NHE1 promoter occurred within 48 hours. This observation suggests that NHE1 promoter activation may be an early event leading to and important in differentiation.

The role of the Na^+/H^+ antiporter in cellular differentiation remains controversial. Earlier studies have shown that intracellular alkalinization accompanies differentiation (32). It has been suggested that there is a causal link between increased antiporter activity and subsequent differentiation (33, 34). Alternatively, the increased antiporter activity may be involved in protection of the cell from acidosis produced during respiratory burst activation (9). However, it has been suggested that in HL 60 cells the activation and increased activity of the Na^+/H^+ exchanger is not a requirement of differentiation (35). The exact role of the protein and its increased expression in differentiation still has to be elucidated. It seems very unlikely that a mechanism of large increases in activity and expression of the protein evolved without significant physiological function. The loss of the absolute requirement for the Na^+/H^+ exchanger in HL 60 cells may be the result of transformation of these cell lines (35). In addition, it could be due to differences between cells grown in culture as opposed to cells growing *in* *vivo*. It has been shown that amiloride analogs that specifically inhibit the Na^+/H^+ exchanger may be useful *in vivo* in inhibition of some types of tumor cell proliferation (36). The exact role of the increased expression of the Na^+/H^+ exchanger in cellular differentiation *in vivo* has yet to be defined.

In addition to activation of the gene our results show that the activity of the Na^+/H^+ exchanger protein is increased in cells treated with retinoic acid. In undifferentiated P19 cells the Na^+/H^+ exchanger protein has been shown to be constitutively activated and unaffected by extracellular stimuli (22). Also, retinoic acid stimulation of HL 60 cells does not appear to act by increasing activity of the Na^+/H^+ antiporter directly. Stimulation by retinoic acid resulted in decreased phosphorylation of the protein despite a rise in internal pH (37). Because differentiation may require either intracellular alkalinization or increased Na^+/H^+ exchange capacity, it may be that the mechanism to fulfill this requirement is increased Na^+/H^+ exchanger gene expression followed by increased protein expression and activity. The early activation of transcription of the gene followed by its decline after the differentiation process, suggests that there is an important role in the process of differentiation itself. Future studies will examine the function of the Na^+/H^+ exchanger in this process.

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CHAPTER FIVE

Regulation of the Na+/H+ Exchanger Gene Expression: Mitogenic Stimulation Increases NHE1 Promoter Activity

INTRODUCTION

The Na^+/H^+ exchanger is a mammalian plasma membrane glycoprotein that mediates the exchange of intracellular H^+ ions for extracellular Na^+ ions with a 1:1 stoichiometry. Several isoforms of the protein have been identified which are designated NHE1-4. The NHE1 isoform is present in all mammalian cells and has been described as the housekeeping isoform of the Na^+/H^+ exchanger family (1). It is involved in pH regulation (2), control of cell volume (3) and is activated by growth factors (4).

The Na^+/H^+ exchanger is involved in cell proliferation and differentiation. During cell proliferation it is responsible for an elevation of intracellular pH that plays a permissive role in growth of some cell types (5). Recent evidence has suggested that mRNA levels of the exchanger are increased during cellular proliferation in intact tissues (6). Hyperplasia of vascular smooth muscle in culture has also been reported to cause up to 25-fold increases in the levels of NHE1 message (7). The role of the Na^+/H^+ antiporter in cellular differentiation remains more controversial. Rao *et al.* (8) examined the regulation of Na^+/H^+ exchanger gene expression and its role in retinoic acid induced differentiation of HL 60 cells. Immediately prior to differentiation of HL 60 cells into granulocyte-like cells, the activity of the Na^+/H^+ exchanger increases and remains elevated well into differentiation (8, 9). There is an 18-fold increase in NHE1 mRNA levels as well as a 7-fold increase in protein levels (8). In addition, we have recently shown that the NHE1 promoter is activated during differentiation of P19 cells (10; Chapter 4, this document). These dramatic changes in Na^+/H^+ antiporter transcription suggest that Na^+/H^+ exchanger gene expression and differentiation are closely linked. However, it is not universally agreed that there is a causal link between increased antiporter activity and subsequent differentiation (11-13).

The mechanisms involved in long-term regulation of the Na^+/H^+ antiporter are only recently being studied. The recent cloning of both the human and the mouse NHE1 genes have enabled more in-depth study of promoter activity (14, 15; Chapter 3, this document). The trans-acting nuclear protein AP-2 has been identified as a transcription factor which can regulate the NHE1 gene (15; Chapter 3, this document). In addition phorbol esters (16) and the transcription factor AP-1 (17, 18) have been implicated in regulation of the NHE1 gene. Glucose is also thought to act through protein kinase C (PKC) to increase NHE1 message levels in certain cell types (19). In this report we characterize mitogenic factors required for induction of expression of the NHE1 gene. We investigate the role of several protein kinases in the regulation of the NHE1 gene and present evidence which demonstrates that the stimulation of cell proliferation causes activation of the NHE1 promoter. In addition, our study indicates that activation of the NHE1 promoter may at least partially be mediated through changes in promoter activity in different phases of the cell cycle.

MATERIALS AND METHODS

Materials

Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim (Laval, Quebec, Canada) and Bethesda Research Laboratories (Gaithersburg, MD, USA). The pBluescript plasmids were from Stratagene (LaJolla, CA, USA). $[^3H]$thymidine was purchased from ICN

Biomedicals (Ontario, Ontario, Canada). All other chemicals were of analytical or molecular biology grade and were purchased from Fisher Scientific (Ottawa, Ontario, Canada), Sigma (St. Louis, MO, USA) or BDH (Toronto, Ontario, Canada).

Cell Culture

NIH 3T3 cells were obtained from Dr. J. Stone of the Department of Biochemistry, University of Alberta. They were maintained in DMEM media supplemented with 10% fetal bovine serum. Where indicated serum was reduced to 0.5% and other components added as described.

Stably transfected cell line

For construction of a stable cell line NIH 3T3 cells were transfected using the calcium phosphate precipitation method as described earlier (15; Chapter 3, this document). Stably transfected cells were transfected using pRSVneo plasmid (1 µg) and pXP-1MP (20 µg). pXP-1MP was constructed as described earlier (15; Chapter 3, this document). The 1.1 kb fragment contains base pairs -1085 to +22 of the NHE1 promoter. After transformation cells were incubated for 24 hours before being replated at a dilution of 1:10. After replating the cells were treated with 400 µg/ml of G418 for 14 days. G418 resistant colonies were identified and individual colonies were repropagated until reaching a higher density. They were then assayed for the ability to express luciferase activity. Polymerase Chain Reaction was used to determine if the entire mouse promoter region from pXP-1MP had been inserted. The primers amplified from the 5' end of luciferase to the 5' end of the insert. NIH 3T3 cells stably transfected with the plasmid pXP-1MP (referred to as 1A7) were used to study regulation of the NHE1 promoter.

Reporter gene assays

The medium was aspirated and the cells washed in 1x PBS-1 mM EDTA. After 15 minutes in 1 ml of PBS-1 mM EDTA they were scraped, transferred to a microcentrifuge tube, and pelleted at 10,000 rpm for 30 seconds. The supernatant was removed and the pellet was resuspended in 50 ml of ice cold lysis buffer (Tris 0.1M pH 7.8, 0.5% NP40 and 1 mM DTT) for 15 minutes. The solution was then pelleted at 14,000 rpm for 10 minutes at 4°C. The supernatant was removed and assayed for luciferase activity. Luciferase assays contained 40 µl of the cell lysate and 100 µl of the luciferase assay reagent (Tricine 20 mM, Magnesium Carbonate 1.07 mM, $MgSO_4$ 2.67 mM, EDTA 0.1 mM, DTT 33.3 mM, luciferin 470 mM, ATP 530 mM, Coenzyme A 270 mM, and BSA 1 ng/ml). Luciferase activity was assayed with an LKB luminometer and normalized to the number of cells and protein content. Results are reported as mean ± SE; where not shown the SE was too small to be displayed. Statistical significance was determined with a t-test.

Flow Cytometry Analysis

Cell cycle compartment analysis was done by flow cytometry with Hoechst 33342 staining. 1A7 cells were used at 70% confluence. Cells were incubated with 20 µM Hoechst 33342 for 1 hr at 37°C. Flow cytometry was with an Epic Elite Flow Cytometer (Coulter Electr. Corp.) equipped with an Innova 90-5 Argon laser for generation of the UV line at 353 nm (200 mW) and an air cooled argon laser (15 mW). The signal from Hoechst was collected by PMT equipped with a 525/40 BP filter.

^{3}H-Thymidine Incorporation

1A7 cells were incubated in 1 μCi/ml of [^{3}H]thymidine along with 0.5 % serum, 1 nM thrombin and 10 μg/ml insulin, 10 ng/ml of EGF, or 10 ng/ml of EGF with 10 μg/ml of genistein. After 24 hours the cells were washed in 1 x PBS and scraped from the dishes. The solution was then centrifuged for 30 seconds at 10, 000 rpm to pellet the cells. After harvest, the cells were resuspended in 0.3N KOH. The DNA and protein were then precipitated with 0.9N HCl/25% trichloroacetic acid solution. The precipitate was compressed at 14, 000 rpm for 10 minutes, the solute decanted, and the precipitate pellet was resuspended in 200 μl of 0.1N KOH. Aliquots of 100 μl were taken and measured for DNA concentration or [^{3}H]thymidine incorporation. The amount of incorporated [^{3}H]thymidine was expressed as cpm/ng of DNA.

RESULTS AND DISCUSSION

The NHE1 isoform of the Na^+/H^+ exchanger family has traditionally been described as the "housekeeping" isoform. Housekeeping genes and proteins are usually thought of as non-responsive to external stimuli. A number of studies, however, have shown that mRNA levels for the NHE1 isoform can be increased by a variety of external stimuli including serum, acidosis, PKC and cell proliferation (6, 7, 16, 20, 21). These findings prompted us to investigate the cellular events which lead to the activation of the NHE1 gene. This work became feasible with the recent cloning of the 5' flanking region of the mouse NHE1 gene (15; Chapter 3, this document). We constructed a permanently transfected cell line which contained the NHE1 promoter directing a reporter gene. This approach allowed us to characterize more precisely the changes in promoter expression by avoiding some of the variability inherent in transient transfections.

To construct a stable fibroblast cell line we used a reporter plasmid containing a 1.1 kb fragment of the mouse NHE1 promoter/enhancer inserted 5' to a luciferase gene (Figure 5-1). We have earlier isolated and cloned this region of the NHE1 promoter and have shown that it contains the necessary elements essential for activity (15; Chapter 3, this document). This plasmid was then stably transfected into the mouse fibroblast cell line, NIH 3T3. The resultant permanently transfected cells, named 1A7, were used in all subsequent experiments. Polymerase chain reaction was used to confirm that the entire 1.1 kb NHE1 promoter region was integrated into the genome of the cells and that it was not interrupted mid sequence. We isolated genomic DNA from cells which could express luciferase. Oligonucleotide primers directed against the 5' end of luciferase and to the 5' end of the mouse insert, amplified the appropriate size DNA fragment (not shown). This confirmed that we had inserted the intact 1.1 kb promoter region into the genome of the NIH 3T3 cells.

Serum deprivation has routinely been used to arrest growth of NIH 3T3 cells and rendering them quiescent (22, 23). Because recent evidence has suggested that mRNA levels of the exchanger are increased in at least some models of cellular proliferation (6) and because of a possible involvement of the Na^+/H^+ exchanger in cell proliferation, we examined the effects of reduction of serum content on the activity of the NHE1 promoter. Mouse NIH 3T3 cells (1A7) were grown to approximately 70% confluency and then grown for 24 hours in medium containing either 0.5% or 10% serum.

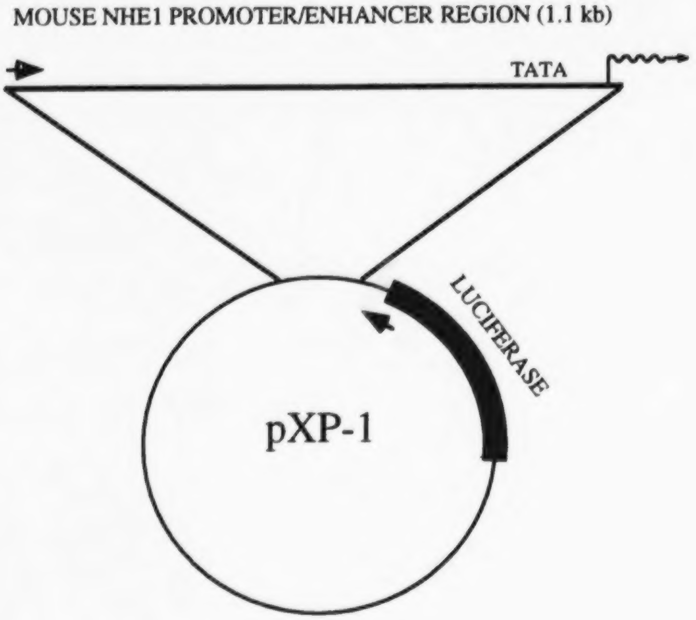
MOUSE NHE1 PROMOTER/ENHANCER REGION (1.1 kb)
TATA
LUCIFERASE
pXP-1

Figure 5-1. **Construction of pXP-1MP**. A 1.1 kb region of the mouse NHE1 promoter was inserted 5' to the luciferase gene of pXP-1. The TATA box is labeled and the region containing the start sites of transcription are indicated by the undulating arrow. The two bold arrows indicate the regions where the PCR primers bind.

Figure 3-2A represents the level of luciferase activity from cells grown in 0.5% and 10% serum. Cells grown in the presence of 10% serum had a 60% increase in NHE1 promoter activity compared to those with 0.5% serum (Figure 5-2A). Also, these cells showed a 50% increase in [^{3}H]thymidine uptake (Figure 5-2B). The effects of serum in these experiments are most likely a result of alterations in the growth state of the fibroblasts. Serum reduced cells undergo a decrease in cellular proliferation, and become quiescent. When serum is re-introduced to these cells, proliferation increases and growth rate rises. These alterations in cell proliferation were reflected in the ability of serum to stimulate DNA synthesis (Figure 5-2B). These results suggest that increased growth rate may result in elevated NHE1 gene expression. Indeed, serum has been shown to be a hyperplastic agonist and to cause large increases in NHE1 mRNA levels in vascular smooth muscle cells (7). It should be recognized, however, that the effects of serum on promoter activity could be direct rather than being mediated through a reduction of cellular proliferation. Serum contains a variety of factors which may affect the activity of this promoters directly. In addition, the NHE1 promoter contains whole or partial consensus sequences for serum response elements and for a number of transcription factors activated by serum including AP-1, AP-2, and Pea3 (15; Chapter 3, this document).

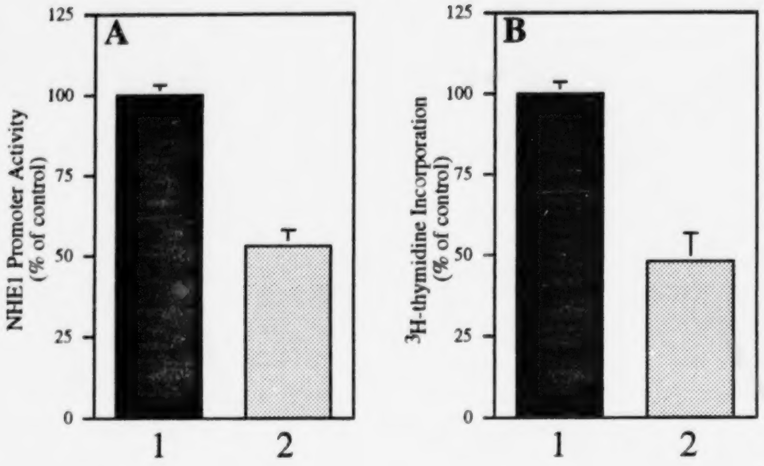

Figure 5-2. **Effect of serum on NHE1 promoter activity in NIH 3T3 cells.** A, mouse fibroblasts were stably transfected with pXP-1MP and grown in media containing either 10% or 0.5% serum (lanes 1 and 2, respectively). The NHE1 promoter activity is expressed as % of control. B, identical experiments were performed as above except for the addition of 1 μCi/ml [^{3}H]thymidine. For both A and B, results are reported as mean ± SE.

To identify some of the specific factors contained in serum which may act to stimulate NHE1 promoter transcription, we treated 1A7 cells with a variety of mitogens. One of these mitogens was epidermal growth factor (EGF). Cells were initially incubated in serum free medium for 24 hours to decrease cell proliferation and to eliminate any residual mitogens. The cells were then treated with EGF for 24 hours, harvested, and assayed for luciferase activity. The results are shown in Figure 5-3A. Lane 1 is the control cells which were not stimulated with any mitogens. Lane 2 represents cells which had been treated with 10 ng/ml of EGF. EGF is a mitogen which is widely recognized to stimulate cell replication (24). The initial events in the action of EGF are the activation of tyrosine kinase with a subsequent effect on a large number of protein substrates (24). Our results with EGF show that this factor produced a 4.75-fold increase in NHE1 promoter driven transcription.

EGF has also been found to stimulate proliferation in a variety of cell types (25-27). This stimulation is a result of the tyrosine kinase activity of the EGF receptor itself (25). How the NHE1 promoter is activated in cells treated with EGF, however, is not known. One possibility is that the increase in proliferation may affect NHE1 transcriptional activity. Also, the tyrosine kinase activity may phosphorylate a yet unidentified transcription factor which can then regulate the NHE1 promoter. To determine the role of tyrosine kinase in this process, the tyrosine kinase inhibitor, genistein, was used. Genistein has been shown to inhibit the mitogenic effects of EGF in many cell types (25-27). Cells were treated as before except the media contained 10 ng/ml of EGF along with 10 µg/ml of genistein. Surprisingly, cells treated with EGF and genistein (lane 3), showed a 4.6-fold increase over cells treated with EGF alone (lane 2) and a 11.4-fold increase over untreated cells (lane 1). These results indicate that genistein can cause increased transcriptional activity of the NHE1 gene. Because genistein inhibits tyrosine kinase activity it is most likely that the inhibition of the kinase results in the eventual increase in NHE1 promoter activity. One possibility may be that genistein increases proliferation thereby stimulating NHE1 transcription. To test this hypothesis, genistein was examined for the ability to stimulate DNA synthesis in NIH 3T3 cells (Figure 5-3B). Cells treated with EGF resulted in increased [^{3}H]thymidine incorporation (lane 2) while fibroblasts which were exposed to EGF and genistein together (lane 3) showed no increase in [^{3}H]thymidine incorporation compared with control (lane 1). The increases in NHE1 promoter activity due to EGF alone may be explained by increases in cellular proliferation. The effects of genistein on the NHE1 promoter in EGF treated cells, however, cannot be attributed to the induction of proliferation.

Since an increase in proliferation was not detected on tyrosine kinase inhibition, some other mechanism must be involved which elevates NHE1 transcription in the presence of EGF and genistein. One possibility may be that the NHE1 gene is down-regulated by a transcription factor that can only bind when phosphorylated by a tyrosine kinase not linked to EGF. When genistein is added to the cells, the phosphorylation of this protein may not occur thereby preventing the binding to the NHE1 gene. Once the protein is removed the NHE1 promoter is no longer under the control of a suppressor and the activity increases. Another possibility explaining the increase in transcription may be that genistein itself can somehow elevate the promoter's activity. Future experiments are necessary to test this hypothesis.

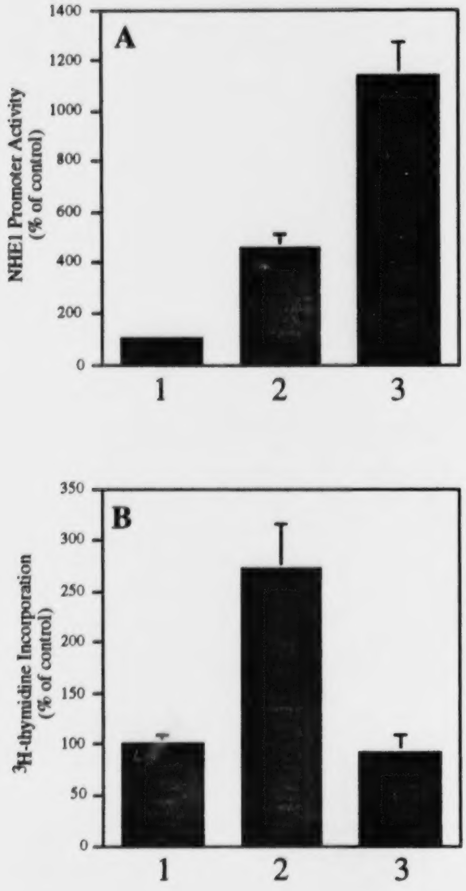

Figure 5-3. **NHE1 promoter activity in mouse NIH 3T3 fibroblasts stimulated with EGF.** Mouse fibroblasts were stably transfected with pXP-1MP and grown in serum free media for 24 hours followed by treatment with EGF for another 24 hours. A, lane 1 represents control cells which have not been treated with any mitogen. Lane 2 represent cells treated with 10 ng/ml of EGF. Lane 3 represents cells treated with 10 ng/ml of EGF along with 10 μg/ml of genistein. B, identical experiments as above were performed except for the addition of 1 μCi/ml [^{3}H]thymidine. For both A and B, results are reported as mean ± SE; where not shown the SE was too small to be displayed.

In an attempt to identify other factors contained in serum which may act to stimulate NHE1 promoter transcription, 1A7 cells were incubated in the presence of thrombin and insulin. Cells were initially incubated in serum free medium for 24 hours to decrease cell proliferation and to eliminate any residual mitogens. The cells were then treated with either thrombin, insulin or thrombin and insulin for 24 hours and assayed for luciferase activity. The effects on the NHE1 promoter activity are shown in Figure 5-4A. When 1A7 cells were treated with 1 nM of thrombin we found a 2.6-fold increase in transcription of the mouse NHE1 promoter (lane 2). Cells treated with 10 μg/ml of insulin (lane 3) produced a 3.2-fold increase in NHE1 promoter driven transcription. Moreover, the addition of both thrombin and insulin together (lane 4) stimulated the NHE1 promoter 3.7-fold. Thrombin has been reported to be a mitogenic stimulus in some cell types, either alone or in conjunction with growth factors (28). Similarly, insulin by itself or in combination with other factors, can stimulate cell proliferation (29). In some cell types the effect of insulin on cell proliferation may be at least partially mediated by the Na^+/H^+ exchanger. Insulin may act to elevate intracellular pH which may be necessary for cell growth depending on the cell type and extracellular pH (29-31). The combination of thrombin and insulin together, however, produced only a slightly elevated level of activation of the NHE1 promoter as compared to insulin alone. This suggests that activation of the NHE1 promoter via thrombin and insulin probably occurs through the same or a closely related pathway as insulin alone. The overall effect of this pathway is most likely the stimulation of cellular proliferation. To demonstrate the ability of these agonists to stimulate proliferation the level of thymidine incorporation during DNA synthesis was measured. Cells treated with thrombin and insulin demonstrated increases in $[^3H]$thymidine incorporation

(Figure 5-4B). This indicates that alterations in the NHE1 promoter activity may result from the stimulation of these cells to proliferate.

One of the pathways involved in the activation of the NHE1 promoter may be mediated by a serine/threonine protein kinase, possibly PKC. We used the inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) to examine the contribution of the thrombin/insulin dependent PKC pathway on the activity of the NHE1 promoter (Figure 5-4A). When cells were treated with thrombin and insulin in addition to H7 (65 μM), the increase in promoter activity was greatly attenuated (Figure 5-4A; lane 5). H7 is an inhibitor of serine/threonine protein kinases with partial specificity to PKC (32). Our results show that H7 can block a large amount of the stimulatory effects of thrombin and insulin (Figure 5-4A; lane 4). This indicates that the increase in NHE1 transcription stimulated by thrombin and insulin can be reduced by inhibition of serine/threonine protein kinases. To provide further evidence that PKC is indeed involved in the thrombin/insulin pathway, another PKC inhibitor was used. Similar to H7, staurosporine blocked the stimulatory effects of thrombin and insulin on the NHE1 promoter activity (not shown). The inhibition of the actions of thrombin and insulin is most likely an effect of altering cellular proliferation.

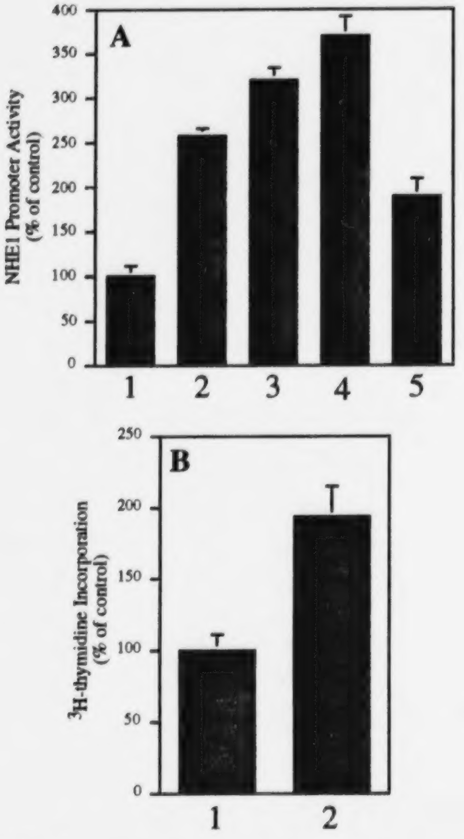

Figure 5-4. **NHE1 promoter activity in mouse NIH 3T3 fibroblasts stimulated with thrombin and insulin.** A, Cells were initially incubated in serum free medium for 24 hours and then split into five groups. Group one was left as control and was not treated (lane 1). Lanes 2, 3, and 4 are groups of cells which had been treated with thrombin (1 nM), insulin (10 µg/ml) or thrombin and insulin (1 nM and 10 µg/ml) respectively. Lane 5 represents treatment with thrombin (1 nM) and insulin (10 µg/ml) plus H7 (65 µM). B, identical experiments as above were with thrombin and insulin (1 nM and 10 µg/ml) except for the addition of 1 µCi/ml [^{3}H]thymidine. For both A and B, results are reported as mean ± SE.

Protein kinase C has been suggested to be involved in regulation of the NHE1 promoter (16, 18). In addition stimulation of PKC acts as a mitogen similar to EGF, possibly working through phosphorylation of mitogen activated protein kinase (33). To test the role of PKC in regulation of the NHE1 promoter, 1A7 cells were subjected to treatment with the phorbol ester, phorbol 12-myristate 13-acetate (PMA). Cells were treated with either 100 nM of PMA or 100 nM of the inactive form, 4 α-phorbol 12-myristate 13-acetate (α-PMA) for 24 hours (Figure 5-5A). We chose to expose the cells to PMA for 24 hours because maximal effects were seen at this time and no effects of down regulation of PKC were found during this time (Figure 5-5B). Cells were also treated with 100 nM PMA in the presence of 65 μM of the inhibitor H7. The results are shown in Figure 5-5A. PMA treatment produced a small but significant increase in NHE1 promoter activity in NIH 3T3 cells. The inactive form of PMA (α-PMA), however, produced no increase in NHE1 transcription. Also, the PKC inhibitor, H7, decreased the effects of PMA completely. The mechanism of action of PKC on the NHE1 promoter is not yet known. Two possibilities exist. Firstly, PKC may be involved in the activation of some transcription factor which can trans-activate the NHE1 gene. The recent identification of AP-2 as a regulator of the NHE1 gene shows the involvement of transcription factors in NHE1 promoter activation (15; Chapter 3, this document). Also, AP-1 can be activated by PKC and this activation has been suggested to be involved in the regulation of the NHE1 gene (18). However, direct evidence for this activation in the intact gene has not been shown. The second possibility for the involvement of PKC in activating NHE1 transcription is through its mitogenic properties. PKC may act indirectly to stimulate the gene similar to other types of mitogenic stimulation (33).

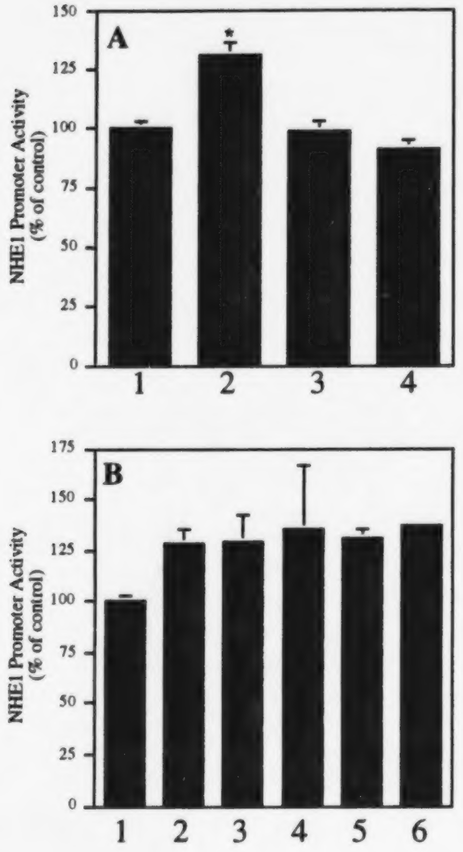

Figure 5-5. **The effect of PMA on NHE1 promoter activity in mouse NIH 3T3 cells.** Mouse NIH 3T3 cells were grown in serum for 24 hours then treated for another 24 hours. A, lane 1 represents control cells which have not been treated. Lane 2, 3 and 4 represent 100 nM of PMA, 100 nM α-PMA, and 65 μM H7, respectively $^*P<0.05$. B, cells were treated with 100 nM of PMA for 0, 2, 4, 8, 24, and 48 hours and assayed for luciferase activity (lanes 1, 2, 3, 4, 5, and 6 respectively). The NHE1 promoter activity is expressed as percentage of control. Results are reported as mean ± SE; where not shown the SE was too small to be displayed.

The modest effect of PMA on the NHE1 promoter activity supports the contention that PKC alone cannot be responsible for direct regulation of the NHE1 gene. A more plausible explanation suggests that mitogenic stimulation of cell proliferation is the cause of elevated NHE1 promoter activity.

The results of experiments with serum, EGF, thrombin, insulin and PMA together demonstrate an activation of the NHE1 promoter which occurs with mitogenic stimulation. This activation may be due to a direct effect of these mitogens on the promoter. These effects, however, are also consistent with activation of the promoter indirectly through stimulation of cell proliferation. Since hyperplasia has been shown to increase steady state levels of NHE1 mRNA (7), we suggest that the promoter is also regulated in a growth-dependent manner. When cells receive mitogenic stimuli a number of immediate-early-response genes are activated (34). Many of these genes are transcription factors and are responsible for the trans-activation of late-response genes. These late-response genes are often directly or indirectly necessary for the onset of DNA synthesis and mitosis (34). Because of the observed increase in NHE1 promoter activity due to mitogenic stimuli, we examined whether an increase in the NHE1 promoter activity could be detected in any specific stage of the cell cycle. Cells were sorted according to the phases of the cell cycle. These groups were identified as early G1, late G1, S and G2 phases. Figure 5-6 represents the levels of NHE1 promoter directed transcription in these groups of cells. In the G1 and S phases of the cell cycle, NHE1 promoter activity is low. When the cells enter G2 phase, however, the cells are preparing to undergo mitosis immediately. It is at this point where we

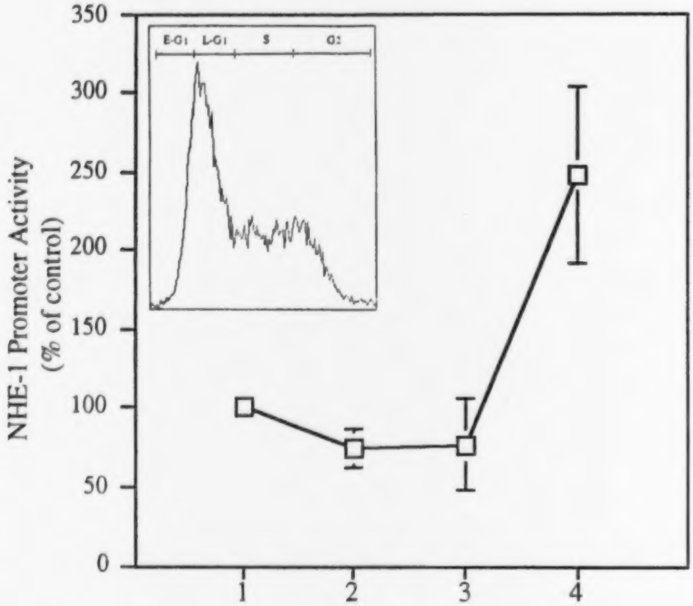

Figure 5-6. **Analysis of NHE1 promoter activity in NIH 3T3 cells separated according to the phases of the cell cycle.** NIH 3T3 cells were stained with Hoechst 33342 dye and separated with flow cytometry. Cells were sorted according to cellular DNA content and divided into early G1 (lane 1), late G1 (lane 2), S (lane 3) and G2 phases (lane 4). The same number of cells from each group were analyzed for luciferase activity. NHE1 promoter activity is expressed as a percentage of early G1. Results are reported as mean ± SE; where not shown the SE was too small to be displayed. The histogram of the cell cycle distribution is inset. The areas divided into the phases of the cell cycle are indicated.

see a 2.7-fold increase in NHE1 promoter activity. These results are consistent with a permissive role of the exchanger in cell proliferation. They support the suggestion that the effects of mitogenic stimulation on the NHE1 promoter may be due to increased cellular proliferation.

The exact role of the Na^+/H^+ exchanger in cell growth and proliferation has not been firmly established. There is evidence demonstrating an important role of cytoplasmic pH regulation in proliferation of normal and tumor cells (35, 36). Moreover, intracellular protons have been reported to be essential during the initiation of mitogenic responses (38). Indeed, the concentrations of H^+ ions themselves can affect the transmission of secondary messengers leading to DNA synthesis and mitosis (38). An early event in the response of most cells to mitogenic factors is often the activation of the NHE1 protein (37). Our study has shown that besides increasing activity of the protein, mitogens also activate the NHE1 promoter during stimulation of cell proliferation. We show that activation of the NHE1 gene is dependent on the growth state of the fibroblasts and occurs in the cell cycle immediately prior to mitosis. We cannot conclude that this activation is essential for the cells to progress through the cell cycle, however, our results are consistent with an important role of the protein in this process. We suggest that alterations in the NHE1 gene activity may play an important role in the control of cell growth and proliferation. Future experiments will investigate this hypothesis.

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CHAPTER SIX

Conclusions and Future Directions

CONCLUSION

The regulation of intracellular pH is important to all living cells. Fluctuations in pH affect growth and proliferation, differentiation and in the myocardium, contractility (1-5). In addition to acute regulation of exchanger activity, our study suggests that long-term regulation of the Na^+/H^+ exchanger may be important in these processes. In the myocardium, acidic conditions induced by ischemia may result in increases in the steady state level of NHE1 transcripts (6; Chapter 2, this document). Furthermore, retinoic acid induced differentiation can also cause increased NHE1 transcription in HL 60 cells and P19 cells (1, 7; Chapter 4, this document). In fibroblasts the NHE1 gene can be induced upon mitogenic stimulation (8; Chapter 5, this document). These examples provide direct evidence to the importance of the long-term regulation of the NHE1 gene in a variety of cell types.

Myocardial ischemia results in acid accumulation within the cell. Studies have suggested that there is a physiologically adaptive mechanism by which some mammalian cell types are able to upregulate the Na^+/H^+ exchanger in response to chronic acid load (9, 10). We report that low-flow ischemia results in increased NHE1 message in the mammalian myocardium. More severe ischemia prevents this increase, suggesting that damaged tissue may not be capable of the ischemic response. The exact mechanism for the increase in steady state mRNA is not known. Elevated mRNA levels are the result of increased transcription, decreased transcript degradation, or a combination of the two effects. To date, there has not been any reports on the stability of NHE1 mRNA. There have, however, been examples demonstrating that increased NHE1 mRNA levels can be a direct result of increased NHE1 transcriptional activity (1, 11).

The complete understanding of the mechanisms involved in the elevation of NHE1 mRNA in the myocardium during ischemia has not been achieved. We have yet to demonstrate that elevated exchanger transcript levels are in fact due to acid accumulation as a result of ischemia. It is possible that some other factor is responsible for this increase. During ischemia there are many events which may directly or indirectly affect the levels of NHE1 mRNA. These include cell swelling, the generation of free radicals, and the accumulation of metabolic products with subsequent activation or inhibition of key enzymes (12, 13). The confirmation that the increases in exchanger message levels are due to decreases in pH, would be an interesting extension of our work. Whether the accumulation of acid is responsible or not, the role of the Na^+/H^+ exchanger in ischemia and reperfusion may be a critical one. There is ample evidence to suggest that amiloride and its analogs can reduce the detrimental effects of reperfusion by inhibition of the antiporter. The task ahead is to determine whether the increased mRNA levels we reported during ischemia are translated into protein and if this protein is helpful or harmful to the myocardium.

In many areas the study of the molecular biology of the cardiac Na^+/H^+ exchanger is still in its infancy. The development of sensitive quantitative assay for both the message and the protein is highly desirable. It would then be possible to examine the antiporter levels in a number of disease states including metabolic and respiratory acidosis, diabetes, and in the ischemic myocardium. However, given the low level of the exchanger, this task may be difficult. There have been no published studies on the regulation of the Na^+/H^+ exchanger gene in the myocardium. The elements of the promoter that are responsible for basal activity in the heart are only now being investigated. The Na^+/H^+ exchanger has been reported to undergo dramatic changes in the level of transcription during differentiation (1) and is involved in cell development and differentiation in some cell types (14-16). Whether the Na^+/H^+ exchanger is important during development of the myocardium is also not yet known. Although these challenges are great, progress in the near future should lead to a greater understanding of pH regulation in the myocardium and an improvement in the ability to treat the diseased myocardium.

With the discovery of long-term regulation of the Na^+/H^+ exchanger many new questions arise as to the elements which are in control of NHE1 transcription (17). The identity of putative cis-acting elements can be suggested with the aid of computer generated programs. Characterizing the trans-acting factors, however, remains more elusive. These nuclear proteins that bind to the DNA and aid in the regulation of transcription are often difficult to identify. To date, we have identified the only transcription factor which is shown to directly bind to and activate the NHE1 gene (18; Chapter 3, this document). Fortunately, this transcription factor is a well characterized trans-acting element known as AP-2. The identification of AP-2 as a transcription factor involved in the regulation of the NHE1 gene makes it clear that nuclear proteins can regulate the NHE1 gene. This information will undoubtedly spur on other research groups to search for more transcription factors which are involved in regulating the NHE1 promoter. We have already discussed the findings with AP-1 and C/EBP and the possibilities of other factors binding to the NHE1 promoter region (19, 20). With the identification of more transcription factors involved in increasing NHE1 transcription, we will gain further insight into how the Na+/H+ exchanger is regulated. Furthermore, the complex processes involved during the interactions of these regulatory events may also be elucidated.

The transcription factor AP-2 can be regulated in a cell-type specific manner and can vary during different phases of cellular development Information already exists which describes the role of AP-2 in transcriptional regulation during some retinoid-affected morphogenic processes (21). AP-2 has also been shown to be present most abundantly in neuroectodermal tissues (21). Similarly, other studies have shown that the level of AP-2 increases substantially in human embryonal carcinoma cell lines during retinoic acid induced differentiation (22, 23). Based on our existing knowledge about AP-2 and the exchanger, we hypothesized that during embryonal carcinoma cell differentiation, elevated AP-2 levels can have enhancitory effects on the NHE1 gene. Our studies on the embryonal carcinoma cell line, P19, confirmed our hypothesis. The exact involvement of AP-2 and the exchanger in differentiation, however, has not been elucidated. Future studies should reveal why NHE1 transcription is increased in this process and whether the Na+/H+ exchanger is necessary for differentiation. It is possible that, as for many cellular processes, there may be an alternate mechanism for regulating pH during differentiation. A 20-fold induction of the promoter and a 3-fold increase in exchanger activity, however, suggest that elevated NHE1 levels play a significant role in differentiation.

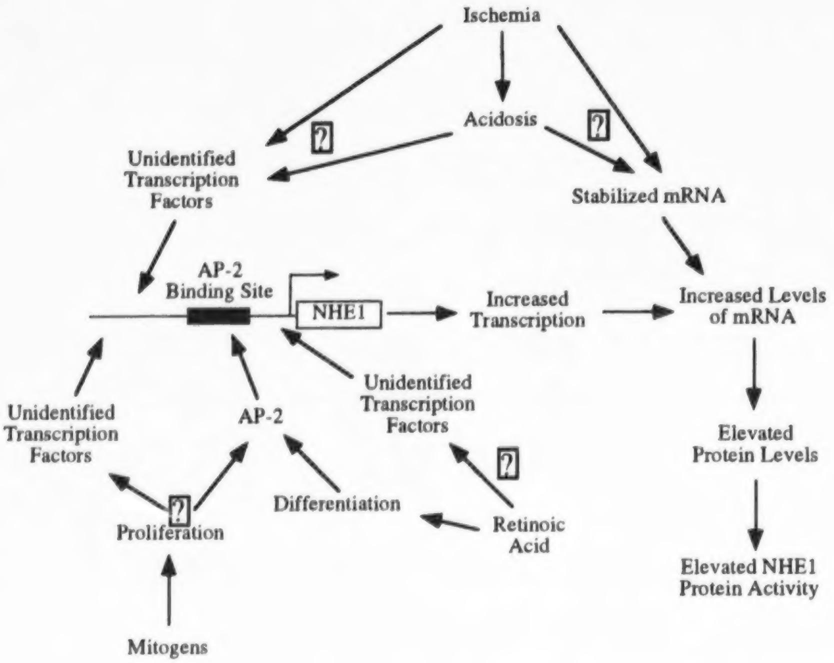
Future studies involving cellular differentiation and the exchanger should involve the differentiation of the P19 cells into a cell type other than neuroectodermal cells. This would indicate if the role of NHE1 in differentiation is universal or if it is specific to cells destined to become neurons. Since AP-2 mRNA levels do not increase during DMSO induced mesoendodermal differentiation, it is unlikely that the AP-2-dependent increase in NHE1 is universal (23). The specificity of this regulation to neuroectodermal cells may be extremely important and should command further attention. Another interesting experiment would involve the identification of the exact mechanism responsible for the upregulation of the NHE1 gene during the direct effect of retinoic acid. We have shown that 5 μM of retinoic acid can directly induce the exchanger gene 4-fold (7; Chapter 4, this document). The mechanism responsible for this regulation is not known. The NHE1 gene does not have a typical retinoic acid receptor binding site present in the 1.1 kb promoter/enhancer region but other proteins may mediate this effect. One such protein is the transcription factor EGR-1. This factor is induced by retinoic acid and is most abundant in the brain (24). Also, the mouse NHE1 gene possesses an EGR-1 site 3' to the AP-2 site. This region of DNA must be studied, however, before any conclusions about EGR-1 can be drawn.

The Na^+/H^+ exchanger has long been suggested to be involved in cell growth and proliferation. During cellular proliferation, the exchanger is responsible for an elevation of intracellular pH. This increase has been suggested to play a permissive role in the growth of certain cell types (2). The long-term regulation of NHE1 has not been examined in detail. Vascular smooth muscle cells subjected to hyperplastic agonists have been reported to result in a 25-fold increase in exchanger mRNA (17). The cause of this increase has not been established. Our results, however, indicate that increased transcription of the NHE1 promoter may be responsible for a portion of this increase in some cell types. When we subjected NIH 3T3 cells to mitogenic factors we reported an increase in promoter activity. Upon further examination it was found that the effects of the mitogens were likely a result of an increase in cellular proliferation. When cell growth was slowed, mitogenic stimulation was able to increase the amount of actively dividing cells. This increase in dividing cells in the population resulted in an increase in NHE1 gene transcription. Cells in G2 phase of cell cycle possessed elevated NHE1 promoter activity. This phase occurs just prior to mitosis and increased exchanger activity may be important for mitosis to occur. Alternatively, increased NHE1 promoter activity may provide elevated levels of exchanger protein which is necessary in another phase of the cell cycle. The reasons for the elevation are still unclear and future research in this area is ongoing.

It should be noted that many of the above conclusions have been drawn from the data derived from the effects of a reporter gene. This assay involves the measurement of a proteins activity to estimate the transcriptional activity of the NHE1 gene. Although this is a widely used technique for studying transcription it has some drawbacks. It is possible that the results we are reporting may be due to luciferase itself, not to effects of the NHE1 promoter. We have, however, performed some experiments under specific conditions using a different promoter to direct luciferase. These studies indicate that our results are due to the specific promoter and not due to the activity of luciferase. Also, the results obtained from transfection experiments may not accurately reflect what we would see in cells in the intact animal.

Future experiments with the intact animal may reveal how accurate our studies have been.

Our studies began with the measurement of NHE1 transcript levels in the myocardium during an event known to cause increased intracellular acidosis. Although the exact method and reason for elevated NHE1 mRNA levels is not known, we have identified a physiological event which increases mRNA levels of the Na^+/H^+ exchanger. We have since progressed to a finer study of how the gene itself is regulated. The identification of AP-2 as one transcription factor involved in the control of NHE1 transcription levels has allowed us to progress even further. With the information we gained from the studies on AP-2 we have identified a specific cellular event which is closely linked to an AP-2 dependent increase in NHE1 promoter activity. This line of research has built upon itself and has culminated with a better understanding of how the NHE1 gene is controlled (Figure 6-1). The indication that the NHE1 gene can be regulated during specific stimulatory events is somewhat opposite to its early reputation as a housekeeping gene. Housekeeping genes are normally thought of as genes which are constitutively expressed at low levels (25, 26). The fact that NHE1 transcription can be regulated by a variety of stimuli is extremely exciting for future research in this area. It has now been shown that there exists another method to regulate Na^+/H^+ exchanger and the processes involved in this regulation are waiting to be discovered. Hopefully, our studies will provide a firm base for future researchers to build upon in their attempts to explain all the events involved in NHE1 gene regulation.


Ischemia
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Stabilized mRNA
AP-2 Binding Site
NHE1
Increased Transcription
Increased Levels of mRNA
Unidentified Transcription Factors
AP-2
Unidentified Transcription Factors
Elevated Protein Levels
?
?
Proliferation
Differentiation
Retinoic Acid
Elevated NHE1 Protein Activity
Mitogens

Figure 6-1. **Model for the Regulation of the Na^+/H^+ Exchanger**. The possible mechanisms of induction of expression of the NHE1 gene are indicated as shown. The NHE1 promoter/enhancer region is indicated by the horizontal line. The black box represents the region of DNA which is capable of binding the transcription factor AP-2 and the start sites of transcription are indicated by the right angled arrow. Straight arrows indicate the temporal sequence of events involved in both NHE1 regulation and expression. Boxed question marks represent possible events involved in the induction of expression of the NHE1 gene.

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